Using Microarrays to Quantify Stress Responses in Natural Populations of Coral

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Using Microarrays to Quantify Stress Responses in Natural Populations of Coral

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TABLE OF CONTENTS

Acknowledgments iii

List of Tables vii

List of Figures viii

Summary xv

Chapter 1. Development of a coral cDNA array to examine gene expression profiles in *Montastraea faveolata* exposed to environmental stress 1

- Abstract 1
- Introduction 2
- Materials and Methods 9
- Results 21
- Discussion 30

Chapter 2. Temporal analysis of gene expression in a field population of the Scleractinian coral *Montastraea faveolata* 41

- Abstract 41
- Introduction 42
- Materials and Methods 44
- Results 48
- Discussion 61

Chapter 3. Profiling differential gene expression of corals along a transect of waters adjacent to the Bermuda municipal dump 69

Abstract 69

Introduction 69

Materials and Methods 71

Results 76

Discussion 80

Chapter 4. Application of a focused microarray to quantify coral stress on South Florida reefs 95

Abstract 95

Introduction 95

Materials and Methods 97

Results 108

Discussion 119

Concluding Remarks 135

Appendix 137

References 167

List of Tables

Table 1.1	The 32 ESTs represented on the cDNA array, environmental conditions associated with isolation of each gene and putative identification determined by BLAST. Accession numbers represent sequences within GenBank that showed significant similarities to the ESTs on the array. †ESTs on the array with their own accession numbers. NS = ESTs with no significant homology to sequences in GenBank.	**14**
Table 1.2	ESTs genereated using RT-PCR and designed primers. Primer sequences are shown in the 5' to 3' direction. F = forward primer, R = reverse primers.	**23**
Table 2.1	Pearson's correlation coefficients for up-regulated genes and corresponding environmental metric.	**59**
Table 3.1	Spot intensities (x ± SE) of control genes on 8 membranes. Six replicates in each category were pooled to yield mean levels of expression. There were no significant differences of expression for any gene ($F_{3,92}$ = 1.3, P>0.05) within site, but there were significant differences for expression levels between sites ($F_{3,92}$ = 11.7, P<0.01). There was also a significant difference in the mean signal intensities of the different control genes (One-way ANOVA, $F_{3,92}$=6.58, P<0.001).	**77**
Table 4.1	Number of genes in each functional category and the grouping of the categories.	**104**
Table 4.2	Deviations from the standard least squares mean (StdLsmean = 0) for a subset of significant genes produced by multivariate ANOVA between samples. The majority of genes (72.5%) fall within |1.0| units of the StdLsmean. Genes that deviate by ~ +/- 1.0 units are highlighted. Red indicates a positive deviation from the StdLsmean (elevated) and green a negative deviation (decreased). § *Indicates no accession number since the gene sequence has not yet been submitted to Genbank.*	**116**
Table A.1	Comprehensive list of oligonuceotide sequences incorporated on the array, the corresponding gene name, accession number, oligo sequence, length, and melting temperature (TM).	**137**
Table A.2	Number of genes from each species represented on the array.	**148**

List of Figures

Figure 1.1 Mean expression of genes exhibiting differential responses to temperatures of 24ºC (ambient), 28ºC or 31ºC. Genes are uPAR - urokinase plasminogen activator receptor, CAN – carbonic anhydrase, Control – average of 3 ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. A single asterisk indicates significant up-regulation ($p<0.05$) and double asterisks indicate significant down-regulation ($p<0.05$) compared to ambient conditions. **25**

Figure 1.2 Mean expression of genes responsive to salinities of 38 ppt (ambient), 43 ppt or 46 ppt. Genes are uPAR - urokinase plasminogen activator receptor, CAN – carbonic anhydrase, TRX – thioredoxin, Control – average of 3 ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. An asterisk indicates significant up-regulation ($p<0.05$) compared to ambient conditions. **26**

Figure 1.3 Mean expression of genes responsive to modified light intensities, PAR (ambient) or UVR. Genes are uPAR - urokinase plasminogen activator receptor, CAN – carbonic anhydrase, Control – average of 3 ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. An asterisk indicates significant up-regulation ($p<0.05$) compared to ambient conditions. **26**

Figure 1.4 Varying gene expression in response to different treatments displayed as blot intensity and visualized using chemiluminescent detection. A representative ribosomal gene is shown to represent the average expression of all three expressed ribosomal genes. **A)** *Temperature*. CAN is down regulated at 28ºC and 31ºC, uPAR is up regulated at 28ºC and down regulated at 31ºC, ribosomal genes exhibit down regulation with elevated temperatures. **B)** *Salinity*. CAN, thioredoxin and the ribosomal genes are up-regulated in response to elevated salinity; uPAR is up regulated at the highest salinity of 46 ppt. **C)** *Light*. CAN and uPAR are up regulated in response to UV; ribosomal genes are also up regulated but to a lesser degree. **27**

Figure 1.5 Northern dot blot compared to cDNA array analysis for uPAR. **A)** Salinity at 38 ppt, 43 ppt, and 46 ppt. **B)** Temperature at 24°C, 28°C, and 31°C. Bars indicate standard error. Mean expression is the average signal intensity of log transformed data. The expression level was not statistically compared between analyses. A single asterisk indicates significant up-regulation ($p<0.05$) compared to ambient (38 ppt or T24°C), double asterisks indicate significant down-regulation ($p<0.05$) compared to ambient. **29**

Figure 2.1 Environmental data collected from NOAA SeaKeys/C-Man station at Long Key for 2001. **A)** Salinity (ppt) and ocean temperature (°C). **B)** Photosynthetically active radiation (PAR, umol/m2/sec) and transmissometry (Formazine Turbidity Units, FTU). **45**

Figure 2.2 Cluster 1. Gene expression levels of three ribosomal proteins with a Pearson's correlation coefficient = 0.99. Sample collection dates in 2001 are indicated on the X-axis and log-transformed expression levels in arbitrary units on the Y-axis. Vertical lines indicate one standard deviation. **51**

Figure 2.3A Cluster 2. Pearson's correlation coefficient = 0.87. A negative value represents a log base 2 transformation of a signal that was <1.0 but still above the background. **52**

Figure 2.3B Gene expression profile of UC1. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Aug 22nd and Sept 5th are significantly elevated compared to dates June 20th and July 18th. **53**

Figure 2.3C Gene expression profile of Thioredoxin. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression levels on April 24th and May 15th are significantly lower than on May 24th. **53**

Figure 2.3D Gene expression profile of uPAR. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression levels on Aug 14 were significantly lower than Apr 24th, Jun 20th, Aug 22nd, Sept 5th, and Sept 26th. **54**

Figure 2.4A Cluster 3. Pearson's correlation coefficient = 0.62. A negative value represents a log base 2 transformation of a signal that was <1.0 but still above the background. **55**

Figure 2.4B Gene expression profile of UC2. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression levels on Aug 22nd and Sept 26th are significantly elevated compared to July 18th. **55**

Figure 2.4C Gene expression profile of TRAP. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression on Sept 26th is significantly elevated compared to June 6th, Sept 5th, and Oct 15th. **56**

Figure 2.4D Gene expression profile of Ferritin. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression on Sept 5th is significantly elevated compared to May 15th and June 6th. Expression on Sept 26th is significantly elevated compared to May 15th, June 6th and Aug 14th. **56**

Figure 2.5A A negative value represents a log base 2 transformation of a signal that was <1.0 but still above the background. There is no data point for UC3 on May 15th since no signal was detected. **57**

Figure 2.5B Gene expression profile of UC3. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Note there was no signal detected on May 15th. **57**

Figure 2.5C Gene expression profile of PUcope17A. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal lines represent 1 SD from the mean for all twelve dates. **58**

Figure 2.6 Gene expression profile of MT. A negative value represents a log base 2 transformation of a signal that was <1.0 but still above the background. This gene's nearest neighbors were Cluster 3. **58**

Fig. 3.1 Map of Bermuda's Castle Harbour and the four sites in this study. The municipal dump in indicated by the red circle. Site 1 is 50 meters from the dump. Site 2 is 960 meters from the dump. Site 3 is 2.73 kilometers from the dump. Site 4, the control site, is located outside of the harbour and is 14.39 kilometers from the dump. **72**

Fig. 3.2 Representative dot blots for all genes expressed at each Bermuda study site. The ribosomal dot blot for each location is actually representative of four separate ribosomal genes. **76**

Figure 3.3 Expression of control genes at all four Bermudan study sites. Control genes expressed at Annex are significantly different in their levels of expression compared to the other sites ($F = 11.7$, $P<0.01$). Expression levels of all four control genes within a location do not differ significantly from each other ($F = 1.3$, $P>0.5$). All values have been normalized to the global average which equals the value of one on the y-axis. **77**

Figure 3.4 Signal comparisons from the cDNA array and Northern dot blots for the copper and uPAR genes. ND indicates a signal was not detected. **79**

Figure 4.1 Map of collections sites produced using GoogleTM Earth. Depths, in meters, are listed after the site in parentheses and GPS coordinates are noted. **98**

Figure 4.2 **A)** Temperature data from January 2005 through June 2006. Data was collected from 20 meters near site 1 using an Acoustic Doppler Current Profiler (ADCP). Mean temperature for each collection are indicated in degrees Celsius. **B)** Precipitation and storm data acquired from the NOAA National Data Center, recorded hourly by the Miami, FL Dade County station (COOPID 85663). Data equals weekly total. Red diamonds are collection dates. Blue triangles are hurricanes Katrina and Wilma, and tropical storm Ophelia. **99**

Figure 4.3 Quantile box plot distributions of a subset of the 72 arrays. Each point represents one array, green dashes are the mean of data within an array, whiskers show the range of the data and the box indicates the 25th, 50th and 75th percentiles. **A)** log base 2 transformed data, **B)** log base 2 transformed and loess normalized data. **106**

Figure 4.4 Deviation of significant genes from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning (NCF), multifunctional (MF), stress response (SR), and symbiont specific (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different cluster. **110**

Figure 4.5 Overview of response by group; normal cellular functioning (NCF), multifunctional (MF), stress response (SR), and symbiont specific (ZOOX) genes. The median values of deviations from the StdLsmean for genes within a group were graphed by sample. Statistical differences between samples were compared by ANOVA. NCF ($p = 0.9970$); MF ($p = 0.8740$); SR ($p = 0.0205$*); ZOOX ($p < 0.0001$*). Correlation analysis compared group responses SR/ZOOX = 0.90; NCF/MF = -0.16; all groups = -0.37. *An alternate version of this graph by cluster is located in the appendix, Figure A.6.* **114**

Figure A.1 Pie graph of variance within the data produced by principal component analysis. Sources of variability in principal components 1 through 3 are 81.99% of the total variance. Residual-74.92% (red), Date-4.47% (green), Site-18.43% (yellow), Colony-2.19% (blue). **148**

Figure A.2 Heat map of Ward's hierarchical cluster analysis. Gene expression patterns between samples of M. *cavernosa* are clustered based on deviation from the StdLsmean produced by the ANOVA between samples. Ten clusters of genes and six clusters of samples with similar profiles were generated. Red indicates a value > StdLSmean, green indicates a value < StdLSmean, and black is not different from the mean. **149**

Figure A.3a **Clusters 1 and 2.** Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample. Cluster 1 = J51, J52, O1, and O2. Cluster 2 = J53 and O3. **152**

Figure A.3b **Cluster 3.** Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample. **153**

Figure A.3c **Cluster 4.** Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample. **154**

Figure A.3d **Cluster 5.** Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample. **155**

Figure A.3e **Cluster 6.** Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample. **156**

Figure A.4 Heat map of Ward's hierarchical clustering analysis of the deviations from the StdLsmean produced by the ANOVA between dates. Nine clusters of genes and two clusters of dates with similar profiles were generated. Red indicates a value > StdLSmean, green indicates a value < StdLSmean, and black is not different from the mean. **157**

Figure A.5 Heat map of Ward's hierarchical clustering analysis of the deviations from the StdLsmean produced by the ANOVA between sites. Eight clusters of genes and three clusters of dates with similar profiles were generated. Red indicates a value > StdLSmean, green indicates a value < StdLSmean, black is not different from the mean. **158**

Figure A.6 Overview of response by group. Genes within functional categories were further grouped based on their overall role in the cell. These groups include normal cellular functioning genes (NCF), multifunctional genes (MF), genes involved in the stress response (SR), and symbiont specific genes (ZOOX). The median values of deviation from the StdLSmeans for genes within a group were graphed by sample. **159**

Figure A.7 Histogram distribution and outlier box plot of the deviations from the StdLsmean for each significant gene produced by the multivariate ANOVA. Most genes (72.5%) fall within +/- 1.0 of the StdLsmean and are indicated by the dark green bars. Outlier genes are indicated by colored dots; blue = normal cellular functioning genes (NCF), grey = multifunctional genes (MF); yellow = stress response genes (SR) and green = symbiont specific genes (ZOOX). **160**

Figure A.8 Volcano Plot. Graph of genes that are significantly different between coral collected from site 2 in August (A2) and coral from site 1, June 2005 (J51). The X-axis is the difference in level of expression of a gene between the samples. The Y-axis is the -log10(p-value) that indicates the degree of significance (higher numbers = greater significance). The color of the circle indicates the functional group: blue = NCF (normal cellular function), grey = MF (multifunctional), yellow = SR (stress response) and green = ZOOX (symbiont specific). The dashed, red horizontal line is the cut-off for the false discovery rate (Q = 10). *Due to the large number of comparisons produced by this analysis (276) this is the only representative graph included in this report.* **161**

Figure A.9 Functional Response. Deviations from the standardized least squares mean (StdLsmean = 0) averaged between significant genes by functional category. Bars indicate standard error. Significant differences between categories are indicated by p-value (Welch ANOVA). The number of genes represented in each category is listed in parentheses after the category heading. Blue bars are categories in the normal cellular functioning group (NCF), grey bars represent multifunctional genes (MF), yellow bars indicate the stress response group (SR), green bars indicate symbiont-specific group (ZOOX). **162**

Summary

Covering less than 1% of the planet surface, coral reefs are one of the world's most valuable ecosystems in terms of ecological, economic and cultural capital (Wilkinson, 2004). Nevertheless, they are declining at an accelerating rate (Hughes et al., 2003; Wilkinson, 2004). Common stressors associated with declining coral health include elevated temperatures, changes in light intensity, sedimentation, and increased exposure to effluent and xenobiotic pollutants (Hoegh-Guldberg 1999; Brown 1997; Gleason, 2001). Traditionally, physiological responses, such as shifts in respiration, photosynthetic efficiency, changes in growth rate, and bleaching have been measured to assess coral health (Anthony and Hoegh-Guldberg, 2003; Jones et al., 1999; Gleason and Wellington, 1993; Fitt and Warner, 1995). These observations are important for identifying coral in serious physiological decline. However, most physiological measurements do not identify the stressor or the underlying molecular mechanisms causing the response. For example, coral bleaching can be caused by many different stressors including elevated temperatures, low salinity and exposure to chemical pollutants. In addition, coral may be stressed beyond recovery by the time a physiological response is observed. Changes in gene expression are key elements of the stress response, usually occur before physiological damage is evident, and can be directly related to the causative agent of stress (Tsuji et al., 2000; Hohmann, 2002).

My research has focused on detecting sublethal responses to stress in Scleractinian coral using genetic biomarkers and gene expression profiling. Through the application of molecular technology, I have developed a focused coral stress gene microarray to

investigate the responses of coral to various stressors. A major advantage of gene expression analysis using array technology is the ability to characterize a likely stressor and identify underlying mechanisms of the response. The temporal and spatial regulation of specific genes within a genome determines the metabolic activity of an organism and can be used to identify changes in cellular responses to various stimuli (DeRisi et al., 1997). These cellular events precede population-level changes and could be useful biomarkers if linked to specific physiological or ecological events.

In chapter one, I describe the development and application of a cDNA array consisting of 32 coral genes that are differentially expressed in response to various stressors. Using various molecular techniques, probes were isolated from *Montastrea faveolata* exposed to extended periods of darkness, continuous sedimentation, elevated temperatures, or increased salinities. These probes were incorporated onto an array and tested against different stressors. Gene expression patterns varied across different stressor treatments, providing preliminary evidence for unique gene expression profiles associated with specific stressors.

The second chapter describes testing the array in a field population to determine the variability of the biomarkers in a natural population across time. This study reveals that targeted DNA arrays can be used to track changes in gene expression in natural coral populations over time. Chapter three examines the gene expression patterns produced by populations of coral at varying distances from a point source of pollution. Responses to heavy metals, sedimentation, and oxidative stress were detected at some locations

sampled in the study. The findings are consistent with the pollutant/stressors reported in other studies as well as my results from laboratory exposures.

The final chapter uses an expanded version of the coral stress gene array, incorporating more coral genes, and is designed for high-throughput analysis. This version has nearly 150 genes involved in the coral stress response as well as normal cellular functions. It is the first study to use a focused Cnidarian microarray to detect stress in field coral populations related to seasonal events, such as precipitation as well as point source stress, such as xenobiotics. Through gene expression profiling, we were able to compare degrees of stress at different sites and times in natural coral populations. In addition, by incorporating symbiont specific genes on the array, we were able to show a clear correlation between the overall expression of symbiont genes with the expression of host stress response genes. This research is important because it identifies stress at a sub-lethal level and can aid resource managers in decision making by prioritizing the stressors impacting coral reefs.

Chapter 1

Development of a coral cDNA array to examine gene expression profiles in *Montastraea faveolata* exposed to environmental stress

Abstract

The utilization of molecular techniques in the field of coral biology has been limited to the application of a few well-known proteins. This paper describes the development of a cDNA array of coral genes that includes both well-characterized and previously unidentified gene fragments and describes the application of the array to investigate changes in gene expression associated with stressful conditions. *Acropora cervicornis* and *Montastraea faveolata* were collected from the Florida Keys and exposed to either natural or anthropogenic stressors to elicit the expression of stress genes for isolation and incorporation onto the array. A total of 32 genes involved in protein synthesis, apoptosis, cell signaling, metabolism, cellular defense and inflammation were included on the array. Fragments of *Montastraea faveolata* were exposed to acute levels of elevated seawater temperature, salinity and ultraviolet light laboratory experiments. Labeled cDNA from the exposed coral was tested against the microarray to determine patterns of gene expression associated with each stressor. Carbonic anhydrase, thioredoxin, a urokinase plasminogen activator receptor (uPAR) and three ribosomal genes demonstrated differential expression across all replicates on the array and between replicate colonies. Specific gene expression patterns produced in response to different stressors demonstrate the potential for gene expression profiling in characterizing the coral stress response.

Introduction

A variety of natural and anthropogenic stressors impact coral reef ecosystems in many regions of the world. Increases in mean annual seawater temperatures (Hoegh-Guldberg, 1999; Buddemeier and Ware, 2003), elevated levels of ultraviolet radiation (Gleason, 2001), and increased salinity fluxes have been recorded on several reefs (Halley *et al*., 1994; Porter *et al*., 1999). Individual organisms respond to changing environments by regulating metabolic pathways to prevent or abate physiological damage. These cellular events precede population-level changes and could be useful biomarkers if linked to specific physiological or ecological events.

Physiological responses such as shifts in respiration (Ferrier-Pages *et al*., 1999; Anthony and Hoegh-Guldberg, 2003), photosynthetic efficiency (Warner *et al*., 1996; Jones *et al*., 1999), changes in growth rate (Ferrier-Pages *et al*., 2000), and bleaching (Lesser *et al*., 1990; Gleason and Wellington, 1993; Fitt and Warner, 1995) have been commonly employed as measures of coral health. In many instances, observations of such responses indicate coral in serious physiological decline. However, most physiological measurements do not identify the stressor or the underlying molecular mechanisms controlling a response. Changes in gene expression and protein production are key elements of the stress response and usually occur before physiological damage is evident (Tsuji *et al*., 2000; Hohmann, 2002). For example, in response to the production of damaging oxygen radicals, genes such as superoxide dismutase, cytochrome c peroxidase, ferritin and thioredoxin are transcribed and translated to bind and neutralize these reactive oxygen species (Cairo *et al*., 1995; Gasch *et al*., 2000). Existing genomic

techniques, developed in biomedical research, are capable of diagnosing and quantifying the impact of stressors on corals. Molecular tools, from the fields of proteomics and transcriptomics, are commonly used to investigate how an individual's genome regulates biological functions. The application of this technology in assessing coral response to environmental change has the potential to advance the field of coral biology and provide valuable management tools for the rapid assessment of coral reef health.

Transcriptomics

All living organisms have thousands to tens of thousands of unique genes encoded in their genome, of which only a small fraction are expressed at a given time. Therefore, it is the temporal and spatial regulation in gene expression that determines metabolic activity (DeRisi *et al*., 1997). The subset of genes transcribed in a given organism is called the transcriptome. It is the dynamic link between the genome, the proteome and the phenotype. Transcriptomics allows the monitoring of differential gene expression which can identify gene function, elucidate the mechanisms behind a biological response, and produce a partial snapshot of cellular machinery in action (Snape *et al*., 2004). To facilitate the discovery of differentially expressed genes, a variety of methods have been developed including differential display PCR (ddPCR) (Liang and Pardee, 1992), subtractive suppression hybridization PCR (SSH PCR) (Diatchenko *et al*., 1996), representational difference analysis (RDA) (Pastorian *et al*., 2000; Hubank and Schatz, 1999), serial analysis of gene expression (SAGE) (Velculescu *et al*., 1995), real-time quantitative PCR (Gibson *et al*., 1996), in situ hybridization (Angerer *et al*., 1987), and hybridization to gene arrays (Larkin *et al*., 2002; Held *et al*., 2004; Voelckel *et al*., 2004).

Although each method has distinct advantages and limitations, the general methodology to detect differentially expressed genes has advanced to automatic high throughput methods such as hybridization-based gene arrays. Thus, to study non-model organisms, such as coral, it is necessary to identify a variety of informative genes and design a cDNA array to functionally characterize the genes and their expression profiles.

Molecular Advances in Cnidarian Research

Applications of well-established molecular methods in cnidarian research are evident and promise to continually expand in the field. For example, genomic information on cnidaria is growing at an ever increasing rate in databases such as the National Center for Biotechnology Information (NCBI) and the European Bioinformatics Institute (EBI). In addition, the Cnidarian Evolutionary Genomics Database, or *Cnidbase* (http://cnidbase.bu.edu), is a publicly accessible database that stores gene expression data for cnidarians such as *Hydra*, the sea anemone *Nematostella*, the stony coral *Acropora*, and the jellyfish *Podocoryne* (Ryan and Finnerty 2003). Although *Cnidbase* focuses primarily on evolutionary changes and structural genomics, it facilitates the functional characterization of newly discovered cnidarian genes by providing a central resource where comparisons of new experimental data can be made with existing data.

Several projects have been initiated to sequence a cnidarian genome. In 2003, a group of coral researchers, using a forum available through NOAA's Coral Health and Monitoring Program (CHAMP), discussed the importance of sequencing a coral genome and selecting a representative species. Based on this discussion, a proposal was made to the

National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH). In addition, The Coral Genomics Group, part of the Comparative Genomics Center at James Cook University in Queensland Australia, has developed a genetic library of expressed sequence tags (ESTs) from *Acropora millepora* in a study of metazoan divergence and evolution (Kortschak *et al*., 2003). Finally, in 2004, the U.S. Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) was reportedly nearing completion of a project to sequence the genome of the cnidarian, *Nematostella vectensis* (Monica Medina, *personal communication*).

Molecular Biomarkers

As of early 2005, the NCBI database held over 150,000 nucleotide sequences and nearly 3000 protein sequences for cnidaria. Scleractinian corals represented over 2% of the nucleotide sequences and 29% of the protein sequences. However, it should be noted that more than half of the sequences are ribosomal or mitochondrial and a significant portion of the remaining sequences are repetitive. Nevertheless, for the development of molecular biomarkers, the database provides a valuable resource for coral researchers. Molecular biomarkers are potentially useful for detecting early signs of change in an organism's physiological state, such as stress or injury due to environmental factors, which may be chemical, physical or biological. Identifying suites of proteins or genes that are expressed in response to specific conditions is the first step in developing a set of biomarkers capable of diagnosing key stressors in an environment.

A few well-characterized molecular biomarkers currently identified in cnidaria, include a metallothionein coding gene (Snell *et al*., 2003), heat shock proteins (Black *et al*., 1995; Sharp *et al*., 1997) and heat shock genes (Gellner *et al*., 1992; Ryan and Finnerty 2003), carbonic anhydrase (Weis and Reynolds, 1999), a ceruloplasm gene (Morgan and Snell, *in prep*), a symbiosis gene (Reynolds *et al*., 2000; Mitchelmore *et al*., 2002), glutathione peroxidase (Schroth *et al*., *in press*) and an oxidative stress protein mRNA (Schroth *et al*., *in press*). Other potential biomarkers involved in developmental regulation (Lohmann *et al*., 1999), evolution (Romano and Palumbi, 1997; Romano and Cairns, 2000), and speciation (van Oppen *et al*., 1999) have also been identified in various orders of cnidaria. It is now technically feasible to incorporate hundreds of coral stress gene biomarkers onto an array and monitor their expression in a single experiment.

cDNA Array Analysis in Ecotoxicogenomics

Once genes of interest are identified and isolated from an organism, they are spotted onto glass slides or nylon membranes for expression profile analyses. A major advantage of gene expression analysis using array technology is the ability to characterize a likely stressor and identify underlying mechanisms of the physiological response. For example, in yeast with a genome of approximately 6200 genes, hundreds of genes are differentially expressed in response to a variety of stresses (Gasch *et al*., 2000). This response has been called the environmental stress response (ESR) and regulates the metabolism of many biochemical pathways for the purpose of preventing damage to membrane structure and maintaining normal cellular functions. In another experiment, Larkin *et al*. (2002) isolated cDNA clones from sheepshead minnow induced by 17-ß-estradiol exposure

using ddPCR. Several estrogen responsive genes including vitellogenin, vitelline envelope protein (ZP2) and the iron transport protein transferrin, along with 17 constituitive genes were spotted onto a nylon membrane. Of the 54 cDNAs spotted on the array, 15 were up-regulated by estradiol exposure, 7 were down-regulated, and 32 were unaffected. Expression profiling using gene arrays has become a powerful tool for comparing stress responses between experimentally exposed organisms and a control population. The ability to identify which stressors are responsible for an observed pattern of expression is a major advantage of this technology.

Toxicogenomics and Ecotoxicology

Molecular tools have been employed by a few researchers to detect stress responses in corals (Black *et al*., 1995; Fang *et al*., 1997; Tom *et al*., 1999; Ammar *et al*., 2000; Downs *et al*., 2000; Morgan *et al*., 2001; Morgan and Snell, 2002). At the present time, most of these responses represent phylogenetically conserved cellular expressions of well-characterized proteins, such as molecular chaperones. The developing field of toxicogenomics examines stress responses at the molecular level with the objective of identifying new patterns of gene expression and/or previously unknown genes in organisms important to ecotoxicology (Snell *et al*., 2003). The application of toxicogenomics in coral research provides opportunities to discover new molecular responses related to specific stressor exposures. Responses at the level of gene expression (transcription) will always precede protein production (translation) of the same transcript. Therefore toxicogenomics offers researchers a tool that is capable of discovering new molecular biomarkers which represent responses that are sub-lethal and/or precede major

physiological events like bleaching. Toxicogenomic investigations also provide coral researchers a direct avenue to access extensive and growing genomic databases supported by well-funded biomedical research. Access to such data offers the possibility of identifying genes based on sequence similarity and suggests potential functions where annotations are available. In addition, such genomic information may help generate new hypotheses to investigate.

In this paper, we describe the development and application of a cDNA array consisting of 32 coral genes that are differentially expressed in response to various stressors. The array is used to investigate changes in transcript abundance in *Montastraea faveolata* responding to controlled exposures of elevated temperature, salinity, and ultraviolet light. Reverse northern analysis, where probes and target are switched in contrast to Northern blot analysis, is used to assess gene expression. Complementary DNA (cDNA), representing the 32 coral genes, is fixed to a membrane (probes). Probes are subsequently hybridized to reverse transcribed and labeled total RNA (targets) isolated from the population being investigated. Chemiluminescent detection can reveal where annealing occurs between a probe and its target (Zhange *et al*., 1996; Dilks *et al*., 2003). In this study, gene expression patterns varied across different treatments, providing preliminary evidence for unique gene expression profiles associated with specific stressors.

Material and Methods

Coral Collections

Fragments of *Montastraea faveolata,* approximately 40 cm^2, were collected at a depth of 4 m from East Turtle Shoal (24º40'N, 80º55'W) in the middle Florida Keys, USA, in April 2001. Conditions on the reef were 25ºC surface temperature and 38 ppt salinity. Coral fragments were transported in closed recirculating containers of natural seawater to the Florida Keys Marine Laboratory (FKML) on Long Key.

Controls and Exposures

Prior to acute stressor exposures, coral fragments were maintained in an outdoor water table with flow through seawater pumped directly from the bay behind the Florida Keys Marine Laboratory. The water on the table was roughly 15 cm deep and ambient light intensity was reduced by 40% with shade cloth. During exposure to temperature, salinity, and light, corals were placed in closed tanks containing 10 liters of natural seawater that was constantly recirculated with a submersible pump. Two coral fragments, from different colonies, were placed in a control tank and treatment tank for each exposure. Manipulations of temperature, salinity and light exposures lasted 4 hours. Sediment and dark experiments were carried out on the water table with 48 hour exposures.

Temperature. Temperature in the control tank was maintained at 25ºC and treatment tanks were elevated to 28ºC and 31ºC, respectively, using an aquarium heater. Salinity in each tank was 40 ppt, which was the salinity of the recirculating natural seawater in the FKML system.

Salinity. Instant Ocean® was mixed with natural seawater collected from the reef to elevate salinity from 38 ppt to 43 ppt and 46 ppt. The control tank was natural seawater at 38 ppt. Temperature in each tank was maintained at 25ºC.

Light. Coral fragments were exposed to either visible or ultraviolet light using fluorescent tubes in the laboratory. Treatments consisted of either two 20 watt daylight lamps (OSRAM Sylvania, Westfield, Indiana) or a combination of one 20 watt UVA (λ_{max} = 368 nm, light emission range = 310-420 nm) and one 20 watt UVB (λ_{max} = 313 nm, light emission range = 270-390 nm) fluorescent lamp (Ultraviolet Resources International, Cleveland, Ohio). All lamps were 60 cm in length and treatments were separated with thick black vinyl to prevent leakage of light to adjacent corals. In both treatments, coral fragments were placed 28 cm from the light source in 38 l aquaria containing 10 l of sea water. Instantaneous measures of irradiance being received by corals in each treatment were determined across a wavelength range of 300-700 nm in 2 nm increments using an underwater scanning spectroradiometer fitted with a cosine collector (LI-1800UW, LiCor Inc., Lincoln, NE). The cosine collector was submerged 2 cm below the water surface and visible and ultraviolet light were recorded in units of Wm^{-2}. These light values are representative of the dose rate being received by the corals in each treatment during the 4 hour exposure. Temperature in the tanks during the exposure period was 26ºC and salinity was 40 ppt.

Sediment. Six coral fragments, two from three different colonies were maintained in the outdoor water table with flow through seawater. Water temperature fluctuated from 24ºC to 28ºC throughout the day and salinity was 40 ppt. Each of the three fragments received 20 mg/cm^2 of sediment twice a day for two days. This rate of application and

sediment load allowed corals enough time to clear most of the sediment before the next application and avoided mortality due to excessive sedimentation. The sediment used was commercial Matt Stone© brand leveling sand (ASTM C-33 standard gradation) and grain size was refined by filtering through a 150 – 250 μm screen.

Dark. Two coral fragments from different colonies were placed in a 20 liter opaque Rubbermaid© plastic container and placed on the outdoor water table with flow-through seawater. Holes were drilled in each end of the container to allow water flow, which was maintained with a submersible pump. Coral fragments were exposed to dark conditions for two days to simulate shading due to suspended sediments.

Extraction of RNA

After exposure colonies were removed from control and treatment tanks and immediately processed for RNA extraction. Most of the coral skeleton was removed with a hammer and chisel, leaving only the top few mm of living tissue. The tissue was ground in 60 ml of a phenol based solution (TRIzol©, Invitrogen™) with a mortar and pestle. Homogenization in TRIzol© stops cellular activity for long-term RNA storage and preservation. Total handling and processing time did not exceed eight minutes. A single 2 ml aliquot from each homogenized coral fragment was isolated and total RNA was extracted following the manufacturer's protocol (based on Chomezynski and Sacchi, 1987). RNA concentrations were estimated by ultraviolet absorbance at 260 nm (Stratalinker 1800, Stratagene) and integrity was confirmed by electrophoresis on a 1% formaldehyde agarose gel.

Target Development

After purification, replicate aliquots of up to 2 ug of total RNA from each treatment were reverse transcribed using SuperScript™ II reverse transcriptase (Invitrogen™) and an oligo (dT) primer (Operon Biotechnologies, Inc.). During reverse transcription, DIG labeled dUTPs (digoxigenin-11-2'-deoxy-uridine-5'triphosphate, alkali-labile; Roche Diagnostics) were incorporated into the transcribed cDNA for subsequent detection using chemiluminescent visualization. To enhance the amplification of longer transcripts in the cDNA pool, the manufacturer's protocol (Invitrogen™) was modified by adding a ramped temperature incubation period. The transcription conditions were 37°C for 1 hour, followed by 42°C or 1 min with a one-degree temperature increase every minute until 50°C for 1 min (Pastorian *et al*., 2000). The reaction was then incubated at 70°C for 15 min to stop the reaction. The resulting cDNAs were quantified with a fluorometer following the manufacturer's protocol (DyNA Quant™ 200, Amersham Biosciences). For each treatment, an aliquot of cDNA was added to a high sodium-dodecyl-sulfate (SDS) buffer (Roche, Indianapolis, IN, USA) resulting in cDNA concentrations of 33 ng per ml (salinity), 47 ng p ml (temperature) and 50 ng per ml (light). These DIG-labeled cDNA solutions were subsequently used as targets to assess changes in gene expression within treatments.

Expression Profiling of Lab Exposed Corals: Array Development

An experimentally designed coral cDNA array was used to evaluate differential gene expression in temperature, salinity and light targets. ESTs on the array represent 32 different cDNAs isolated from *Acropora cervicornis* and *Montastraea faveolata* exposed

to various natural and anthropogenic treatments. These gene fragments were isolated using subtractive hybridization, differential display PCR, or reverse transcription PCR with designed primers (Snell *et al*., 2003).

- *Probes from Previous Studies*

Xenobiotic and heavy metal probes on the array were isolated by Differential Display PCR (ddPCR) in laboratory experiments using *Acropora cervicornis* (Morgan *et al*., 2001; Morgan and Snell, 2002). Corals were exposed for four hours to treatments of copper (25ug/l and 50ug/l), mercury (5.0ug/l and 50ug/l), permethrin (1ug/L and 10ug/l), dibrom (0.5ug/l and 5.0ug/l), or naphthalene (50ug/l and 300ug/l). In addition, a metallothionein probe was also developed using the 50 ug/l copper treatment and reverse-transcription PCR (Snell *et al*., 2003). These probes range in size from 150 bp to ~500 bp. Each probe demonstrates various degrees of response specificity to a small suite of stressors. Most probes show no significant homologies to sequences in GenBank, with the exception of metallothionein isolated from 50ug/l copper (Snell *et al*., 2003), and ceruloplasm, isolated from 50ug/l naphthalene (Morgan and Snell, 2005) (Table 1.1).

Table 1.1. The 32 ESTs represented on the cDNA array, environmental conditions associated with isolation of each gene and putative identification determined by BLAST. Accession numbers represent sequences within GenBank that showed significant similarities to the ESTs on the array. †ESTs on the array with their own accession numbers. NS = ESTs with no significant homology to sequences in GenBank.

Clone	E-value	Exposure	Putative ID	Accession #
S(B) H5	BlastN: 1e-171	Sediment	Ribosomal RNA	AY026375.1
S(C) A6	BlastN: 1e-134	Sediment	Ribosomal RNA	AY026365.1
S(C) C9	BlastX: 2e-19	Sediment	Thioredoxin	CAA76654.1
S(C) E7	TblastX: 5e-59	Sediment	Ribosomal RNA	AY026375.1
S(C) E9	BlastX: 7e-21	Sediment	Trap-D	S59869
S(D) B11	TBlastX: 1e-65	Sediment	Ribosomal	AY026375.1
Dk(C) F1	BlastN: 7e-34	Darkness	Ferritin	CAC84555.1
Dk(C) G10	*NS*	Darkness	uncharacterized	*NS*
Dk(C) G11	RPS-Blast: 7.6	Darkness	uPAR	CDD|14821
T1	BlastX: 1e-22	Thermal: 28°C	Met-aminopeptidase	AAM61284.1
T2	*NS*	Thermal: 28°C	uncharacterized	*NS*
T3	*NS*	Thermal: 28°C	uncharacterized	*NS*
T4	*NS*	Thermal: 28°C	uncharacterized	*NS*
T5	BlastX: 5e-09	Thermal: 28°C	NALP	AAO18165.1
T6	*NS*	Thermal: 28°C	uncharacterized	*NS*
T7	*NS*	Thermal: 28°C	uncharacterized	*NS*
C30	*NS*	50 µg/L Copper	uncharacterized	BI534458†
P22	*NS*	1µg/L Permethrin	uncharacterized	*NS*
Dbs	*NS*	0.5µg/L Dibrom	uncharacterized	BI534457†
Dbl	*NS*	0.5µg/L Dibrom	uncharacterized	BI534456†
H30	*NS*	5 µg/L Mercury	uncharacterized	BI534459†
N40	TblastX 4e-29	50 µg/L PAH	ceruloplasmin	AF08256.7
Mt 1	BlastX: 0.87	50 µg/L Copper	Metallothionein	AAF22486.1
CAN con 60	*NS*	Thermal: 25°C	uncharacterized	*NS*
CX sal 19A	BlastX: 1e-4	Salinity: 43 ppt	ankyrin	gi 28373837 pdb 1N0R A
PU sal 17B	TBlastX: .073	Salinity: 43 ppt	Poly Ubiquitin	TVU27577
DNAG T28 19A	*NS*	Thermal: 28°C	uncharacterized	*NS*
HX sal 17C	*NS*	Salinity: 43 ppt	uncharacterized	*NS*
GST con 10A	BlastX: 1e-07	Thermal: 25°C	Bombesin-like peptides	NM_015548.2
CAN T28	BlastX - C: 8e-04	Thermal: 28°C	Carbonic Anhydrase	AAD32675.1
CX T28 19A	BlastX: 2e-29	Thermal: 28°C	Phosvitin	AAA98791.1
PU cope 17A	BlastX: 3e-13	Thermal: 25°C	Poly Ubiquitin	BAA09860.1

- *Recently Isolated Probes*

Probes on the array derived from exposures to natural stressors were isolated from *Montastraea faveolata* with suppressive subtractive hybridization PCR (SSH PCR) and reverse transcription PCR using designed primers (Snell *et al*., 2003).

Suppressive Subtractive Hybridization PCR. Clontech's PCR-Select™ cDNA Subtraction Kit (Cat# K1804-1) was used to perform two different subtractive hybridizations on coral fragments from the sediment or dark treatment and the control. Total RNA was extracted with TRIzol® (Invitrogen) and mRNA was purified with Oligotex™ (Cat# 72022, Qiagen). Messenger RNA concentrations were estimated by ultraviolet absorbance and integrity was confirmed by gel electrophoresis. After the final round of PCR to enrich for differentially expressed sequences (subtracted product), cDNA libraries were constructed of sequences up-regulated in response to sedimentation and darkness exposures. The subtracted PCR products were shotgun cloned into a vector (pCR® II-TOPO, Invitrogen, Carlsbad, CA, USA), and recombinant plasmids were inserted into competent *E. coli* cells and stored at -80°C in 15% glycerol solution.

Differential screening was performed following the manufacturer's protocol to eliminate false positives (PCR-Select™ Differential Screening Kit, Cat# K1808-1, Clontech). Random clones were picked from the subtracted cDNA libraries, a colony PCR was performed and amplified cDNA inserts were spotted onto nylon membranes following the Hybond-N+ protocol for dot blotting nucleic acids (Amersham Pharmacia Biotech, Inc.). DIG labeled dUTPs (digoxigenin-11-2'-deoxy-uridine-5'triphosphate, alkali-labile; Roche Applied Science) were incorporated into subtracted and unsubtracted cDNAs

during a PCR using primers provided in Clontech's SSH PCR kit. Conditions consisted of 20 cycles at 94°C for 10 sec, 68°C for 30 sec and 72°C for 1.5 min, ending with a 5 min extension step at 75°C. Nylon arrays consisting of the subtracted ESTs were hybridized to DIG-labeled subtracted and unsubtracted cDNA targets following the protocol described in Morgan *et al*. (2001, 2002). Membranes were wrapped in clear plastic and exposed to CL-Xposure Film (Pierce Biotechnology, Inc.) for 16 hours. Dark spots on the film, corresponding to specific transcripts in the target cDNA pool, provided visual identification of expressed probes. A difference in the level of expression of a transcript between subtracted and unsubtracted samples was quantified using the program ImageJ (National Institute of Health). The background around each spot was measured repeatedly and the average measurement calculated from the spot intensity. An EST was considered differentially expressed if it was at least two-fold darker in the subtracted cDNA pool.

Reverse Transcription PCR. A bioinformatics approach was used to isolate several other genes incorporated on the final array. Consensus sequences of well characterized stress-induced genes from several animals were identified in GenBank (National Center for Biotechnology Information) and aligned using the program ClustalW (European Bioinformatics Institute). Primers of 20 – 25 base pairs were designed from regions of high similarity with the program Jellyfish v3.0 (LabVelocity). Total RNA was isolated from *M. faveolata* fragments exposed to previously described treatments and quality was checked on a 1% formaldehyde gel. A RT-PCR kit (Titan One Tube RT-PCR Kit, Roche Applied Science) and a pair of designed primers were use to reverse transcribe RNA and

PCR amplify the resulting cDNA in a single step. The conditions consisted of an incubation period at 45°C for one hour followed by 2 minutes at 92°C. Cycling parameters included 35 cycles of 30 s at 92°C, 45 s at the appropriate annealing temperature for the primer pair and 68°C for 1 minute. Amplified cDNA was separated and visualized on a 2% agarose gel.

Bands corresponding to the correct sequence size were purified using a QIAquick™ gel extraction kit (Qiagen). The extracted product was re-amplified to check for the possibility of co-migrating bands. PCR parameters consisted of incubation at 94°C for 2 min, followed by 25 cycles of 20 s at 94°C, 45 s at the appropriate annealing temperature and 68°C for 1 minute. A 7 min period at 68°C finalized the reaction and completed the sequence extension. Agarose gel electrophoresis confirmed that a single band was amplified and the PCR product was cloned into a vector (pCR® II-TOPO, Invitrogen). The recombinant plasmids were inserted into competent *E. coli* cells and stored in a glycerol solution.

Probes isolated with SSH PCR and RT-PCR were sequenced at the University of Georgia Molecular Genetics Facility (Athens, Georgia USA) and characterized based on sequence similarity to known genes. Searches were carried out using a basis local alignment tool (BLAST), available on-line at NCBI (www.ncbi.nlm.nih.gov). Table 1.1 provides information on the isolated ESTs and indicates putative IDs for those with significant sequence similarity to a known gene.

Membrane & Probe Preparation

Plasmids containing the 32 gene specific probe inserts were isolated from *E. coli* stocks using a commercial kit (QIAprep Spin Miniprep Kit, Cat# 27104, Qiagen). Inserts were PCR amplified using plasmid specific primers, M13 forward and M13 reverse. The conditions consisted of a denaturing step at 94°C for 45 seconds followed by 25 cycles of 95°C for 15 seconds and 50°C for 3 minutes. The amplified inserts were visualized on a 2% agarose gel and quantified by visual comparison with a DNA size/mass ladder. The probes were prepared in 0.2M NaOH and incubated at 37°C for 15 minutes. Denatured cDNA probes were blotted in triplicate onto BioBond™ Nylon membranes (Sigma-Aldrich, USA) in 2 ul volumes at concentrations ranging from 0.25 ug/ul to 0.5 ug/ul. Membranes were neutralized in 0.5M Tris-HCl and cDNA was fixed to the membrane by UV crosslinking.

Three separate PCRs were performed for each replicate probe on a membrane, and all membranes were blotted on the same day. Thus, replicate spots *across* membranes came from the same PCR pool and were identical, while replicate spots *within* a membrane came from different PCR pools and could vary in concentration. The average of replicate spots on a membrane, representing a single probe, was compared between membranes, but replicate spots were not compared within a membrane. This method may create variability in the degree of expression between replicate spots on a membrane, but it controls for false results produced by anomalous polymerase chain reactions.

cDNA Hybridization

Resulting arrays were hybridized with DIG-labeled targets to visualize which probes were expressed in the total RNA from each treatment. The hybridization protocol is described in Morgan *et al*., 2001 and Morgan and Snell, 2002. After overnight exposure of the membranes to x-ray film, dark spots appeared that corresponded to expressed transcripts in the target cDNA pool. A difference in the level of expression of a transcript between control and exposed samples was compared. Hybridizations were performed at least two times using labeled cDNA from different colony fragments in each exposure. Membranes were used only once to ensure a consistent correlation between spot intensity and transcript concentration.

Array Analysis

Densitometry of blots was performed using ImageJ software (NIH). Measurements were recorded from replicate blots for every detectable gene on each membrane. Control genes were identified by homology to rRNA protein sequences (BLASTX 2.2.9, NCBI nr database). Background signals were quantified by measuring an area around each spot that represented twice the diameter measured within an individual spot. The intensity of each signal was initially determined by subtracting its adjacent background values. Expression of an individual gene was determined by average signal intensities of the three replicate spots of each gene on each membrane.

In order to compare signal intensities of multiple spots on different membranes, all data were log transformed. This manipulation is considered a valid approach for data analysis

where the effects in the data are believed to be multiplicative (Kerr *et al*., 2000). Analysis of variance (ANOVA) was then performed since it is capable of systematically estimating the normalization parameters on all relevant data (Kerr *et al*., 2000). Lavene's Test for Equality of Variance was applied to determine whether compared populations exhibited similar variances. For multiple comparisons where variances were similar, the Student-Neuman-Keuls post hoc test was performed to determine which populations were significantly different. Since ANOVA is generally insensitive to heteroscedasticity, Tamhane's T2 posthoc test was applied (Tamhane, 1979) to multiple comparisons where equal variances were not assumed. In experiments with only one stressed condition (UV and PAR), a Student T-test was performed to compare expression levels between control and exposed populations.

Northern Blot Confirmation

To confirm gene expression results detected on the array, Northern dot blots were performed for the uPAR gene. Total RNA was isolated from coral fragments exposed to temperature and salinity treatments. RNA concentrations were estimated by ultraviolet absorbance and integrity was visually confirmed by agarose gel electrophoresis. One microgram of total RNA from each treatment was blotted onto positively charged nylon membranes (Hybond-N+, Amersham Pharmacia Biotech Inc.) and cross-linked by exposure to UV light. Plasmids containing the uPAR inserts were isolated from *E. coli* stocks (Qiagen QIAprep kit) and amplified using M13 primers. PCR conditions were 94°C for 1 min followed by 20 cycles of 94°C for 15 sec, 52°C for 15 sec and 72°C for 30 sec ending with a 72°C extension step for 5 minutes. Amplified cDNA was visualized

on a 2% agarose gel. The band corresponding to uPAR was excised and purified from the gel (Qiagen QIAquick™ Gel Extraction kit) and the purified cDNA was DIG labeled using conditions described above for amplification of the plasmid insert (Roche Diagnostics). The amplified uPAR gene was quantified by ultraviolet absorbance and the incorporation of DIG labeled bases was confirmed by gel electrophoresis. Hybridization of the uPAR probe to the RNA dot blot array was performed and the results visualized on x-ray film. Differential gene expression was determined by densitometry measurements of the dot blot signals. Statistical tests (previously identified) were performed to compare differences between uPAR expression in treatment and control RNA.

Results

cDNA array composition

The first version of the coral cDNA array, as used in this experiment, includes thirty-two expressed sequence tags representing ribosomal genes and genes expressed in response to xenobiotic and natural stressor exposures. The ESTs range from approximately 150 to 600 bases in length and were isolated from *Acropora cervicornis* or *Montastraea faveolata*. Location on the membrane of each EST probe was determined randomly. However, to prevent spatial biases, adjacent spotting of replicate probes was avoided (Machl *et al*., 2002).

Putative identification of ESTs

Various BLAST programs were used to search genomic and proteomic databases for significant sequence similarities. Forty-four percent of the coral ESTs showed significant

similarities (E-values $<10^{-4}$) to database sequences and putative identifications were assigned (Table 1.1). Another thirteen percent of the ESTs had E-values $>10^{-4}$ and putative IDs were assigned only after it had been determined that conserved amino acids were identified in positions characteristic of a particular protein. The remaining 33% of ESTs showed no significant similarities to database sequences searched in June 2004. All of the genes represented on the array were isolated from corals that had been exposed to some type of stressor; therefore it is not surprising that coral genes with significant e-values from BLAST would be similar to stress-induced genes in other organisms.

- ESTs from RT-PCR

Database search results of the sixteen cDNAs amplified using designed primers produced no significant sequence similarities for eight of the cDNAs (unknown), five sequences showed similarity to non-target genes, and three sequences showed similarity to the genes of interest (Table 1.2). Several factors play a role in the amplification of non-target sequences. Primers must be very specific for the desired template to be amplified. Cross reactivity with non-target DNA sequences results in non-specific amplification of DNA. In addition, excess DNA polymerase and low annealing temperature can result in mispriming. The PCR primers designed for this study were specific for the targeted genes based on similarities between sequences from well-characterized organisms in GenBank (NCBI). The relatively low specificity of some of the primers may be due to the limited diversity of cnidarian sequences in the databases at the time the primers were designed. However, the amplification of non-target sequences is not necessarily a negative result, since novel and interesting genes were identified (Harris *et al*., 2004).

Table 1.2. ESTs generated using RT-PCR and designed primers. Primer sequences are shown in the 5' to 3' direction. F = forward primer, R = reverse primers.

Clone	Primer Sequences 5' to 3'	Amplified Product
T1	(F) catggatgtgtcgcagttc (R) ggagcaatgaatcctccagt	Met-aminopeptidase (*non-target*)
T2	(F) gctgccagaaattacaaaggat (R) ggtcaaatgggtttccctct	*uncharacterized*
T3	(F) gatgctgtcgctgttacaatg (R) tcatgccttccacagtttc	*uncharacterized*
T4	(F) atgaaagaggtagccgaagc (R) acgagaaccacgtcatgga	*uncharacterized*
T5	(F) agaggaataatatcaagctgt (R) acctttccactttcttg	NALP (*non-target*)
T6	(F) aagatgaacaggtcacagt (R) tctggatatggaactcct	*uncharacterized*
T7		*uncharacterized*
CAN con60	(F) gatggctgcaccaaaatggg (R) aaagaagccctgacgttgcgt	*uncharacterized*
CAN T28		Carbonic Anhydrase (***target***)
CX sal19A	(F) acatgcatggcaatgaag (R) caactttcacaagatgattgt	Ankyrin (*non-target*)
CX T2819A		Phosvitin *(non-target)*
PU cope17A	(F) aagacactcaccggcaag (R) accttccttatcttggatctt	Poly Ubiquitin (***target***)
PU sal17B		
DNAG T2819A	(F) agtgtctttacttagcagga (R) tcacccaaatgaatgtgt	*uncharacterized*
HX sal17C	(F) acctggacgaaaggattca (R) ttgatgctctcacgatccac	*uncharacterized*
GST con10A	(F) atctccagatatttggcc (R) cgttagtaaaagcgttgaaa	Bombesin-like peptides (*non-target*)

Gene Expression Profiles in Controlled Conditions

Gene expression changes were compared in *M. faveolata* exposed to different intensities of temperature, salinity, or UV stress. To estimate within treatment variance, all probes were spotted in triplicate on a membrane and at least two membranes were used for each exposure. Genes with significantly different average intensities ($p<0.05$) between treatments were considered differentially expressed. Of the 32 genes screened, six exhibited significant differential expression. Three ribosomal genes were consistently expressed but showed slight variation in response to treatments. The remaining three genes (carbonic anhydrase, thioredoxin, and a urokinase plasminogen activator receptor, uPAR) varied to a much greater extent in response to treatments. Descriptions of these genes can be found in Table 1.2.

Baseline expression of control genes

The expression level of control genes, three 28S large subunit ribosomal fragments, was averaged (per treatment) and compared across seven different membrane hybridizations. Baseline conditions of 24°C water temperature, 38 ppt salinity, and PAR were used to make comparisons to laboratory induced stressors. Statistical analysis (Univariate ANOVA, SPSS Inc.) of log transformed control gene expression data revealed unequal variances for the control genes expressed on each membrane ($P<0.05$, Lavene's Test Equality of Error Variances). Since equal variances were not assumed, Tamhane's T2 posthoc test was applied and revealed there were no significant differences ($P>0.05$) in individual membranes or in the expression of individual control genes.

Expression of control genes under stressed conditions

Expression levels of control genes differed significantly under various stress conditions. Corals exposed to 46 ppt salinity showed elevated expression of control genes (Figure 1.2, Figure 1.4, $P<0.05$, Tamhane's T2 test) compared to 38 and 43 ppt salinity exposures. Corals exposed to UV stress also demonstrated significant up-regulation of these genes (Figure 1.3, Figure 1.4, $P<0.001$, Student's T-test). By comparison, these same control genes exhibited a different trend for corals experiencing thermal stress where their expression decreased significantly with increasing temperature (Figure 1.1, Figure 1.4, $P<0.05$, Tamhane's T2 test).

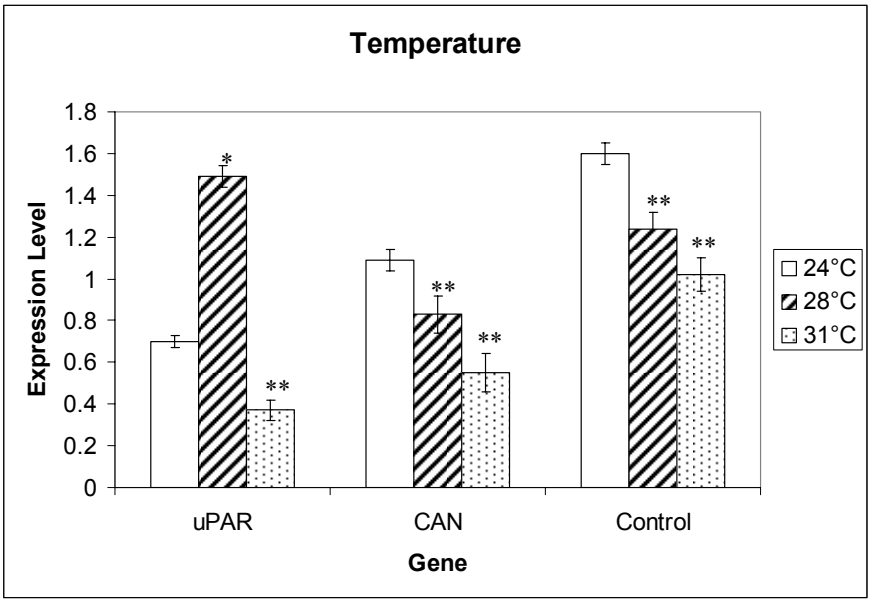
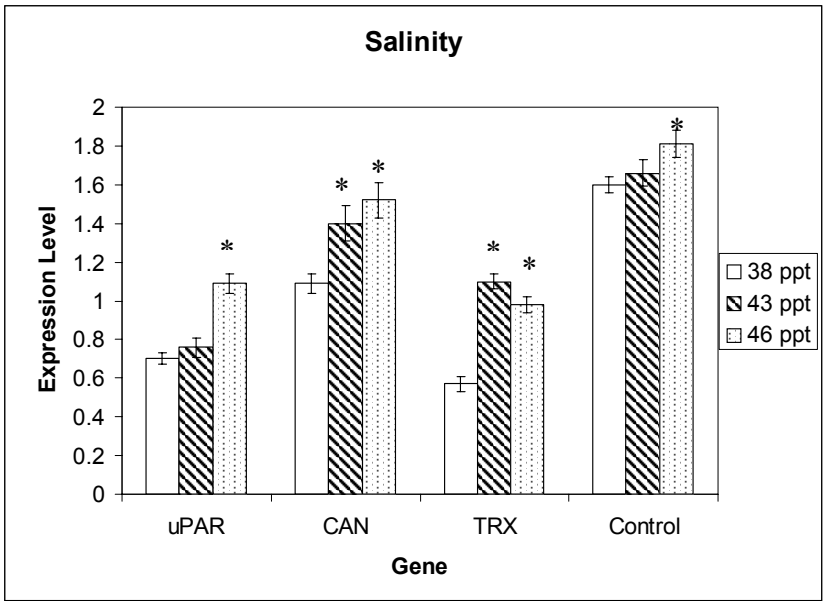
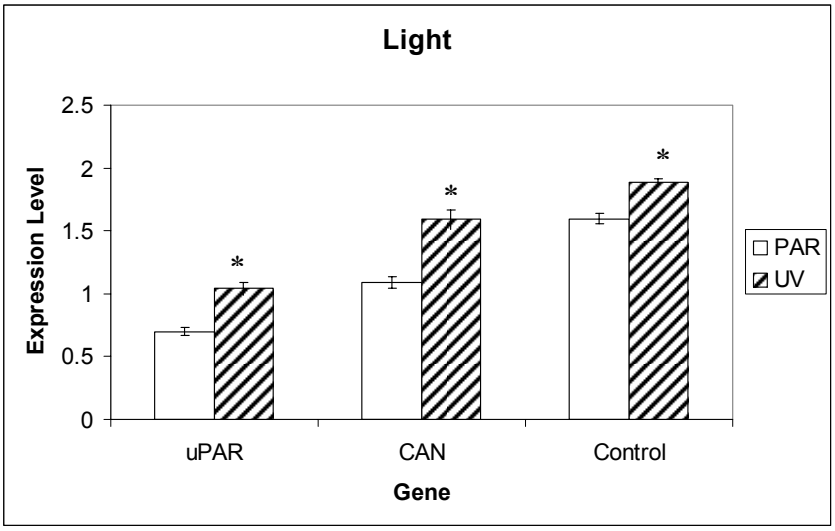


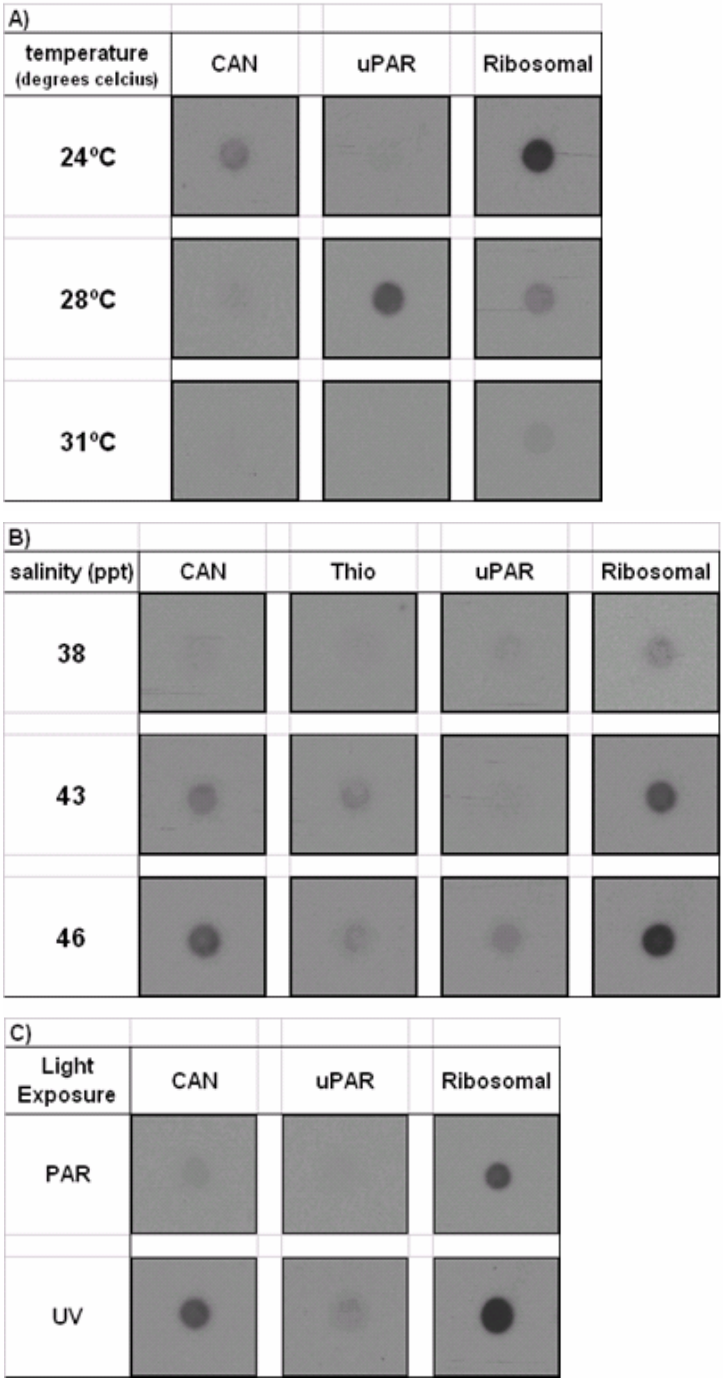
Figure 1.1. Mean expression of genes exhibiting differential responses to temperatures of 24ºC (ambient), 28ºC or 31ºC. Genes are uPAR - urokinase plasminogen activator receptor, CAN – carbonic anhydrase, Control – average of 3 ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. A single asterisk indicates significant up-regulation ($p<0.05$) and double asterisks indicate significant down-regulation ($p<0.05$) compared to ambient conditions.



Figureure 1.2. Mean expression of genes responsive to salinities of 38 ppt (ambient), 43 ppt or 46 ppt. Genes are uPAR - urokinase plasminogen activator receptor, CAN – carbonic anhydrase, TRX – thioredoxin, Control – average of 3 ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. An asterisk indicates significant up-regulation ($p<0.05$) compared to ambient conditions.



Figureure 1.3. Mean expression of genes responsive to modified light intensities, PAR (ambient) or UVR. Genes are uPAR - urokinase plasminogen activator receptor, CAN – carbonic anhydrase, Control – average of 3 ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. An asterisk indicates significant up-regulation ($p<0.05$) compared to ambient conditions.



Figureure 1.4. Varying gene expression in response to different treatments displayed as blot intensity and visualized using chemiluminescent detection. A representative ribosomal gene is shown to represent the average expression of all three expressed ribosomal genes. A) *Temperature*. CAN is down regulated at 28ºC and 31ºC, uPAR is up regulated at 28ºC and down regulated at 31ºC, ribosomal genes exhibit down regulation with elevated temperatures. B) *Salinity*. CAN, thioredoxin and the ribosomal genes are up-regulated in response to elevated salinity; uPAR is up regulated at the highest salinity of 46 ppt. C) *Light*. CAN and uPAR are up regulated in response to UV; ribosomal genes are also up regulated but to a lesser degree.

Expression of stress genes under stressed conditions

The array detected different expression profiles for corals exposed to elevated salinities, temperature shock treatments, or UVB. Three genes (Thioredoxin, Carbonic Anhydrase, and uPAR) each demonstrated distinctively different patterns of expression.

-*Temperature shock*

Carbonic anhydrase showed significant decreases in expression at each temperature ($P<0.05$, Tamhane's T2 test). Expression of uPAR showed significant up-regulation at 28°C as well as significant down-regulation at 31°C ($P<0.05$, Student-Neuman-Keuls). Thioredoxin was not up-regulated under these laboratory controlled conditions (Figure 1.1, Figure 1.4).

-*Elevated Salinities*

Thioredoxin (TRX) and carbonic anhydrase (CAN) were both significantly elevated at salinities 43 and 46 ppt ($P<0.05$, Student-Neuman-Keuls). Expression of uPAR was significantly elevated only at 46 ppt ($P<0.05$, Student-Neuman-Keuls) (Figure 1.2, Figure 1.4).

-*UV exposure*

Carbonic anhydrase showed significant up-regulation in expression after exposure to UV ($P<0.001$, Student T-test). In a similar pattern, uPAR also showed significant up-regulation ($P<0.05$, Student T-test). Thioredoxin was not up-regulated under these laboratory controlled conditions (Figure 1.3, Figure 1.4).

Northern dot blots

A Northern dot blot was performed to verify results obtained using the cDNA array. The uPAR gene was up-regulated in the 28°C and 31°C temperature treatments compared to the 24°C control treatment ($P<0.05$, Student-Neuman-Keuls). At the elevated salinities of 43 and 46 ppt, uPAR expression levels were significantly up-regulated compared to the 38 ppt control treatment ($P<0.05$, Student-Neuman-Keuls) (Figure 1.5).

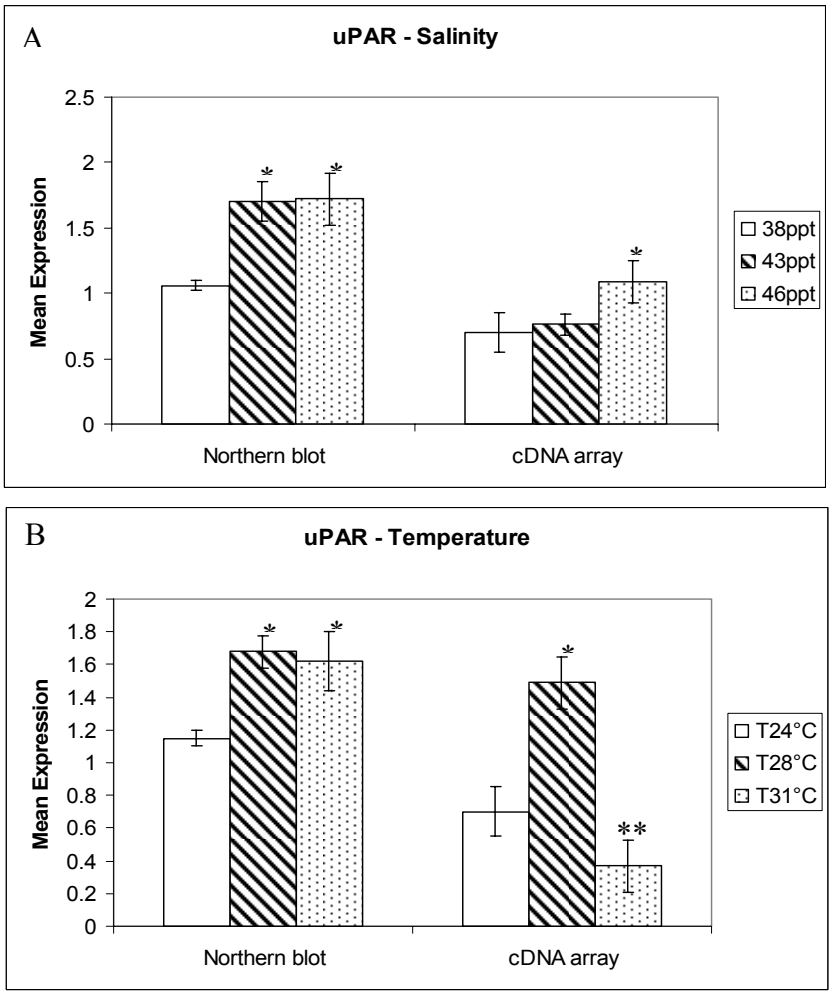


Figure 1.5. Northern dot blot compared to cDNA array analysis for uPAR. A) Salinity at 38 ppt, 43 ppt, and 46 ppt. B) Temperature at 24°C, 28°C, and 31°C. Bars indicate standard error. Mean expression is the average signal intensity of log transformed data. The expression level was not statistically compared between analyses. A single asterisk indicates significant up-regulation ($p<0.05$) compared to ambient (38 ppt or T24°C), double asterisks indicate significant down-regulation ($p<0.05$) compared to ambient.

Discussion

Organisms react to environmental change by altering the expression of suites of genes within a repertoire of thousands of genes in the genome. Changes in gene expression can be general, as in the induction of chaperones in response to a variety of stressors (Hofmann *et al*., 2002), or specific such as a gene that is only expressed upon exposure to an organophosphate insecticide (Morgan and Snell, 2002). The expression pattern derived from a combination of general and specific genes can be interpreted to indicate the effect of stress on cellular functions (Gasch *et al*., 2000; Williams *et al*., 2003).

Differential Expression

Each treatment evaluated in this study resulted in a unique gene expression pattern due to the up- or down- regulation of a subset of genes on the coral array. Thioredoxin (TRX) was only expressed during salinity stress, indicating some degree of specificity in the response. Studies using yeast show that TRX is superinduced during hyperosmotic shock (Gasch *et al*., 2000; Posas, 2000). In addition, TRX acts as a singlet oxygen quencher and hydroxyl radical scavenger maintaining redox balance within eukaryotic cells (Das and Das, 2000). The absence of expression by TRX in other treatments in this study could be due to insufficient exposure time or dose.

A general stress response was demonstrated by elevated expression of the uPAR gene in all treatments. The highest temperature exposure was an exception where all genes, including ribosomal controls, were significantly down-regulated. Pepper *et al*. (1993)

demonstrated that uPAR, which is involved in proteolysis, wound healing and inflammation, was significantly up-regulated in mechanically wounded endothelial cells.

Carbonic anhydrase demonstrated variable expression by increasing upon exposure to elevated salinity and UV, but decreasing when exposed to thermal stress. CAN plays an important role in symbiotic cnidarian respiration (Weis and Reynolds, 1999) and photosynthesis (Weis, 1993; Furla *et al.,* 2000; Estes *et al*., 2003), as well as acid-base balance, ion regulation, and osmoregulation in marine organisms (Wheatly and Henry, 1992; Whiteley *et al*, 2001; Wilson *et al*, 2002). Gilbert and Guzman (2001) demonstrated that activity levels of carbonic anhydrase decreased in anemones exposed to metal contamination. In contrast, carbonic anhydrase activity correlates positively with densities of zooxanthellae and light intensity (Weis, 1991; Weis and Reynolds, 1999).

Temperature

The effect of temperature on corals is widely studied and has been shown to impact photosynthetic efficiency (Warner *et al*., 1996; Jones *et al*., 1998), reduce respiration (Nystrom *et al*., 2001), induce oxidative stress (Lesser 1997), impair CO_2 fixation in symbionts (Jones *et al*., 1998), and alter the configuration of membrane lipids (Tchernov *et al*., 2004). Additionally, temperature has long been ascribed as one of the major causes of coral bleaching (Gates *et al*., 1992; Brown 1997; Hoegh-Guldberg, 1999). In this study, acute exposure of *M. faveolata* to elevated temperatures caused a significant decrease in carbonic anhydrase mRNA, while the uPAR-like gene increased expression at mildly elevated temperatures but returned to background levels at the highest

temperature. Down regulation of gene expression may be related to the observed decreased in ribosomal function at the highest temperature. The repression of ribosomal protein genes and genes involved in a variety of other cellular functions has been observed during the stress response in yeast (Gasch *et al*., 2000). Well-characterized thermal genes were not observed during this experiment. This could be due to a maximum temperature exposure of only 31°C. Most studies using protein biomarkers have not reported the expression of heat shock elements at temperatures lower than 33°C (Black *et al*., 1995; Sharp *et al*., 1997).

Salinity

Elevated salinity affects the ionic regulation (Dietz *et al*., 1997), acid-base balance (Whiteley *et al*., 2001) and natural osmoregulation of an organism (Whiteley *et al*., 2001; Wilson *et al*, 2002). In yeast, hyperosmotic shock causes cell wall and cytoskeleton reorganization (Slaninova *et al*., 2000). At the molecular level, yeast cells increase expression of genes in the high-osmolarity glycerol (HOG) pathway (Hohmann, 2002), and superinduce other environmental stress response genes, including oxidoreductases, cytosolic catalase, and Cu, Zn-superoxide dismutase (Gasch *et al*., 2000; Garay-Arroyo *et al*., 2003). In addition, the induction of molecular chaperones during osmotic stress has been demonstrated in several organisms (Smith *et al*., 1999; Spees *et al*., 2002). Coral exposed to varied salinity concentrations reveal alterations in respiration, photosynthesis, and total protein content (Moberg *et al*., 1997; Ferrier-Pages *et al*., 1999). Ferrier-Pages *et al*. (1999) demonstrated that colonies of *S. pistilata* maintained at an elevated salinity of 40 practical salinity units (psu) died during a 3-week exposure. To date, no studies

have used specific biomarkers to investigate the effects of changes in salinity on cnidaria. All genes observed in this study, including CAN, uPAR, TRX and ribosomal controls, exhibited up-regulation in response to acute hypersaline conditions.

Ultraviolet Radiation

Exposure to ultraviolet radiation (UVR) leads to protein damage, tissue inflammation, DNA damage, and cell death either directly or by generating reactive oxygen species (Miralles *et al*., 1998, Lesser *et al*., 2001). The formation of oxygen radicals disrupts protein synthesis and damages cell membranes resulting in decreased photosynthetic rates (Lesser, 1996; Shick *et al*., 1996). Organisms respond by up-regulating suites of genes that code for transcription factors, growth factors, and proteases, which have been characterized in mammals as the UV response (Devary *et al*., 1992; Miralles *et al*., 1998). The up-regulation of genes that initiate DNA repair or apoptosis has been reported in fish exposed to UVR (Lesser *et al*., 2001), while human epithelial cells induce the expression of uPAR mRNA (Marschall *et al*., 1999). Marine organisms defend against solar radiation by producing natural UV-absorbing sunscreens, identified as mycosporine- like amino acids (MAAs) (Shick and Dunlap, 2002). In addition, the accumulation of antioxidants, such as carotenoids, which quench oxygen radicals, offers protection from the harmful effects of ultraviolet radiation (Mobley and Gleason, 2003). In this study, acute exposure of *M. faveolata* to UVR significantly increased transcription of CAN and uPAR genes.

Verification

Results from the Northern blot verification were consistent with the cDNA array data in that a general trend was apparent. In both analyses, uPAR, was up-regulated exclusively in treatment conditions as compared to ambient conditions (Figure 1.5). This was consistent for both salinity and temperature exposures. Discrepancies between the results can be attributed to differences in sensitivity of the techniques. It has been documented that Northern blot analyses are more sensitive than cDNA array analyses (Taniguchi *et al*., 2001; Chuaqui *et al*., 2002; Dieck *et al*., 2003). However, both techniques produce a degree of experimental variability. The cDNA array results were not completely identical with that of the Northern blot results, but there were clear parallels between the two analyses. This demonstrates that cDNA arrays provide quantitative data, but underscores the importance of validating results with a more sensitive method.

Putative identification of ESTs

Each EST on the coral array may have multiple roles. Based on annotations from homologs, classifications emerge that group some of the coral genes into multiple functional categories. One subset of genes on the array could be classified as representative of oxidative stress (TRX, Cp, Ferritin, Mt). The translocon associated protein-γ (TRAP-D), although not considered an antioxidant, has recently been shown to form cytotoxic aggregates with mutant forms of superoxide dismutase (Miyazaki *et al*., 2004), which is a powerful antioxidant. Another functional subset of genes may be associated with inflammation (Cp, NALP, uPAR). Mullen (2004) points out that inflammation is not well-studied in stony corals, but amoebocytes are believed to be

involved. However, an inflammatory response has been qualitatively and quantitatively characterized in at least one gorgonian (Mezzaros and Bigger, 1999). Additionally, histological examinations of coral tissue show evidence of tissue remodeling under stressed conditions (Mullen *et al*., 2004), which may be related to inflammation. A third subset of genes are induced upon exposure to UV light (TRAP-D, uPAR, TRX, Mt, Poly-U). While not grouped with other genes, the Bombesin-like peptide receptor is worth noting since its function appears to be involved with modulating stress in order to maintain homeostasis (Moody and Merali, 2004)

Strengths and limitations of this technology

Like any technology, cDNA array analysis has certain limitations. Manipulation during hybridization and image processing can add variability to the results. Such sources of fluctuation include the efficacy of reverse transcription, efficiency of target labeling, non-uniform PCR amplification of probes, accuracy of spotting, spot morphology and production of background on the array (Schuchhardt *et al*., 2000; Cook and Sayler, 2003). Biological variability can also affect results. Genetic heterogeneity, previous exposure to stress, and differences in symbiont communities may mask changes associated with the stressor in question. Replication and validation increase the reproducibility of an experiment. Taking replicate samples from the same colony and multiple coral colonies estimates biological variability, while incorporating multiple spots of the same gene on duplicate arrays permits the statistical removal of experimental variability. Most variability is eliminated by proper experimental design and robust

statistical analyses, such as log transformation and ANOVA (Kerr *et al*., 2000; Nadon and Shoemaker, 2002).

The validity of a hybridization experiment depends on the sensitivity, specificity, and reproducibility of results. Sensitivity refers to the ability to detect a single probe out of a population of target cDNAs and to reliably determine a difference in expression between samples. It is directly dependent on the amount of target used in the hybridization mix (Bertucci *et al*., 1999), and is positively correlated with the length of a probe (Zhou and Thompson, 2002). Several studies have demonstrated that cDNA array technology provides good specificity and reproducibility (Schena *et al*., 1995; DeRisi *et al*., 1997; Brutsche *et al*., 2001) even between different array systems (Bertucci *et al*., 1999). For example, Larkin *et al*. (2003) showed that gene arrays are sensitive enough to detect changes in gene expression of a contaminant at environmentally relevant concentrations. Sensitivity can be maximized by starting with quality RNA, optimizing RT-PCR, improving target labeling and optimizing probe length.

Specificity refers to the proportion of probes that correctly hybridize to target sequences and is dependent upon hybridization stringency, sequence identity, and probe length (Zhou and Thompson, 2002). High stringency conditions reduce cross-hybridization events significantly. Miller *et al*. (2002) examined cross-hybridization between five closely related genes. Hybridization stringency was high (0.6XSSC at 68°C) and probes consisted of full length clones. Cross-hybridization between genes from different families was not observed unless sequence identity exceeded 94%. A similar study using four of

the same gene families and less stringent conditions (0.1XSSC at 45°C) observed cross-hybridization at 80% sequence identity (Evertsz *et al*., 2001). Thus, conditions can be adjusted to achieve a broad range of detections. The hybridization conditions in our study were very stringent (0.2XSSC at 68°C), so cross-hybridization to non-target genes is probably inconsequential.

Probes on the coral stress gene array were isolated from different coral species (*M. faveolata* and *A. cervicornis*). It is important to know whether the hybridization system being employed has the ability to identify altered gene expression across species. Several studies have shown cross-reactivity between species in microarray experiment. For example, human cDNA microarrays have been used to investigate cross-species hybridizations in pig (Moody *et al*., 2002), monkey (Chismar *et al*., 2002) and bovine (Adjaye *et al*., 2004) RNA with the majority of genes generating highly reproducible data. Cross-hybridization is expected between species with low genetic diversity. For example, there is approximately 5% sequence difference between the genomes of humans and rhesus monkeys (Chismar *et al*., 2002). Research has shown low genetic diversity between Anthozoan (van Oppen, 1999; Shearer, 2002) suggesting that cross-hybridization between coral species is feasible. In addition, a study by Morgan *et al*. (*in press*) has shown differential expression in *Diploria strigosa* using the coral stress array. In our experiment, 6 of the 32 genes on the array exhibited reproducible expression. The lack of expression by other genes could be due to the absence of the probe in the target cDNA pool, the target was not expressed, low sequence identity, or high stringency conditions. Finally, even though we have focused on the response of the holobiont,

detection of symbiont genes utilizing this array was most likely minimal. BLAST results of probe sequences did not reveal significant similarities to any known plant genes. Cross-reactivity between cnidarian and zooxanthellate genes and between genes of different coral species should be investigated further.

In spite of the limitations outlined above, cDNA array technology is a powerful tool for investigating complex gene expression relationships. The biological function of a gene determines when and where it is expressed, and deviations from homeostasis cause changes in the level of transcription of many genes. Therefore, detailed information regarding the state of an organism can be gleaned from patterns produced by the expression of multiple genes. cDNA arrays provide a practical way of examining the expression of multiple genes in a single experiment. The application of this technology to coral research can facilitate the rapid screening of coral health in the field and identify molecular mechanisms responsible for an observed physiological response. The isolated and arrayed sequences in this study are potential genetic biomarkers of stress, which reveal specific gene expression patterns in response to different conditions. Utilizing even a small suite of biomarkers can direct attention appropriately and fuel future projects that investigate coral stress using integrated technologies.

Future Studies

The cDNA array used in this study represents the first version of a coral stress microarray developed by our lab. Future versions of the array will be robotically spotted on glass slides and include a minimum of ten replicates per gene. In addition, our lab has currently

isolated and characterized approximately 15 new sequences for incorporation onto the array. More sensitive protocols will complement this technology, such as robust quantification of PCR probes and digital detection of gene expression. A series of positive and negative controls will be spotted on the array and spiked controls will be added to the labeled target cDNA. Finally, the standards set by the minimum information about a microarray experiment (MIAME) will be incorporated into all future experimental designs (Brazma *et al*., 2001).

Future studies investigating coral gene expression should address the relationship between exposure time and dose response. Gene transcription in response to a stressor may be transient or sustained depending on the type of stressor and level of exposure. Experiments will be carried out to compare gene expression patterns between acute and chronic exposures of the same stressor. In addition, field experiments will be performed to determine if responses in the laboratory correlate with those in the field. Tests comparing species specificity and cross-reactivity with symbiont genes will be conducted and incorporated into experiments addressing responses by the holobiont. Finally, using integrated approaches, questions of whether gene expression detected by the cDNA array translates into protein production can be addressed. This technology is based on changes in gene transcription. It does not consider the effect of stress on translation, post-translational modification, protein localization, or protein degradation. However, gene expression changes that are not associated with altered protein production are still interesting and relevant, especially if these changes represent reliable, sensitive, and selective markers of a response to specific conditions (Pennie *et al*., 2000).

The possibility of diagnosing coral in the field at sublethal levels of exposure and in multi-stressor environments raises the issue of interpreting gene expression patterns. The accurate interpretation of gene expression may only be possible when experiments are conducted as part of an integrated approach to understand observed responses at the physiological or biochemical levels. There is a vast amount of information already available on the molecular mechanics of the stress response, particularly in yeast (Gasch *et al*., 2000). Experiments should be designed to make the most of these data. Directed research using array technology in conjunction with other proven methodologies can produce new fundamental knowledge about coral biology and response to environmental factors. This study represents an initial attempt at characterizing gene expression profiles in coral exposed to multiple levels of different stressors using a cDNA array and demonstrates how analyzing gene expression could be useful in interpreting the coral stress response.

Chapter 2

Temporal analysis of gene expression in a field population of the Scleractinian coral *Montastraea faveolata*

Abstract

Organisms maintain homeostasis and abate cellular damage by altering gene expression. Coral colonies have been shown to produce unique gene expression patterns in response to different environmental stimuli. In order to understand these induced changes, the natural variation in expression of genetic biomarkers needs to be determined. In this study, an array of genes isolated from Scleractinian coral was used to track changes in gene expression within a population of *Montastraea faveolata* from April to October 2001 in the Florida Keys. The profiles of genes observed in this study can be divided into two groups based on expression over this time period. In spring and early summer, May through July, most of the genes show little deviation from their average level of expression. In August and September, several genes show large deviations from their average level of expression. The physiological and environmental triggers for the observed changes in gene expression have not yet been identified, but the results show that our coral stress gene array can be used to track temporal changes in gene expression in a natural coral population.

Introduction

It has been well documented that coral populations around the world are in serious decline (Done, 1992; Harvell et al., 1999; Wilkinson, 2000). Decreased recovery from bleaching events, increased susceptibility to disease, impacts on reproduction, lowered diversity and death are some of the physiological responses to factors impacting coral populations. Molecular ecology is a rapidly expanding field of biology that is concerned with applying molecular techniques to address traditional ecological questions. The use of genomic technology, such as DNA arrays, can identify coral responses to environmental change before physiological decline is evident (Snell et al., 2003). The unique expression of a specific suite of genes can provide insight into the molecular mechanisms involved in an organism's response to its environment. Edge et al. (2005) review the use of molecular genetic technology as a method to diagnose coral health.

Organisms alter the expression of specific genes in order to maintain homeostasis and abate cellular damage. For example, coral colonies produce unique gene expression patterns in response to different environmental conditions (Edge et al., 2005). In order to understand these induced changes, the natural variation in expression of genetic biomarkers needs to be investigated. While most of the gene expression studies of non-model organisms are conducted in controlled laboratory conditions, field studies are becoming more common (Wiens et al., 2000; Bais et al., 2003; Morgan et al., 2005). However, few of these studies have investigated the natural variation of gene expression within a population (Lejeusne et al., 2006). In order for gene array technology to be a useful tool for detecting population responses in coral, it needs to be determined whether

changes in gene expression can be detected above the natural variation in expression within a population over time (Klaper and Thomas, 2004).

The analysis and interpretation of changes in gene expression by Scleractinian coral may pose challenges not encountered in the study of model organisms due to their colonial morphology, ability to reproduce sexually and asexually, and the blurred distinction between species (Knowlton et al., 1997). In addition, gene expression within a single cell varies in complexity and activation (Levsky and Singer, 2003; Oleksiak et al., 2004; Raser and O'Shea, 2005). Some genes are static, exhibiting little variability in expression over time and under different environmental conditions. Other genes exhibit stochastic expression, fluctuating unpredictably over time in response to a variety of conditions. However, inducible genes fluctuate in a predictive manner in response to specific cues from the extracellular or intracellular environment. The expression patterns produced by a suite of these inducible genes incorporated onto an array can provide information on how a population responds under different conditions.

In this study, I used an array of genes isolated from coral and tracked changes in gene expression in a population of coral through time. Results show that the targeted DNA array can be used to detect changes in gene expression above natural variation in expression within a field population of coral. Genes whose expression did not change are also interesting. During the time sampled, genes responsive to organopesticides and other xenobiotics were not induced.

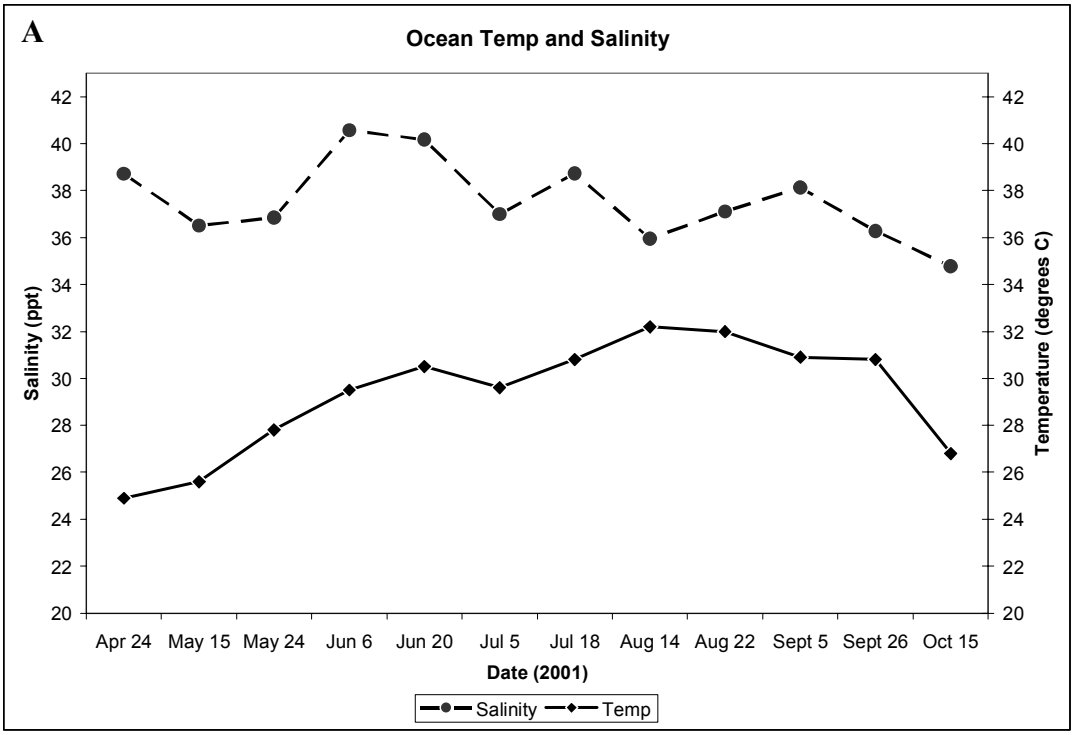
Materials and Methods

Coral Collections

Fragments of *M. faveolata*, approximately 2 cm^2, were collected from five colonies at a depth of 4 m from East Turtle Shoal (24°43'15"N, 80°55'50"W) in the middle Florida Keys, USA, in 2001. Samples were collected twice a month during a seven month period (April to October) with the exception of a single collection in late April and early October. Coral fragments were transported to the Florida Keys Marine Laboratory (FKLM) on Long Key in closed containers of natural, recirculating seawater. Samples were then processed for subsequent molecular analysis. Excess skeleton was removed with a hammer and chisel, and the samples were ground in 25 – 30 ml of a phenol based solution (TRIzol®, Invitrogen™) with a mortar and pestle. Homogenization in TRIzol stops cellular activity for long-term storage and preservation of samples used in molecular analyses.

Environmental Data

Environmental parameters including ocean temperature (degrees Celsius), salinity (ppt), photosynthetically active radiation (PAR, umol/m2/sec), and transmissometry (Formazine Turbidity Units, FTU) for April through October, 2001 were downloaded from NOAA's SeaKeys/C-MAN database recorded by the station at Long Key (LONF1, 24° 50' 24" N, 80° 51' 36" W). If environmental data was not available for a collection date in this study, then a calculated mean consisting of two dates before and two dates after the particular date was used (Figures 2.1A and 2.1B).



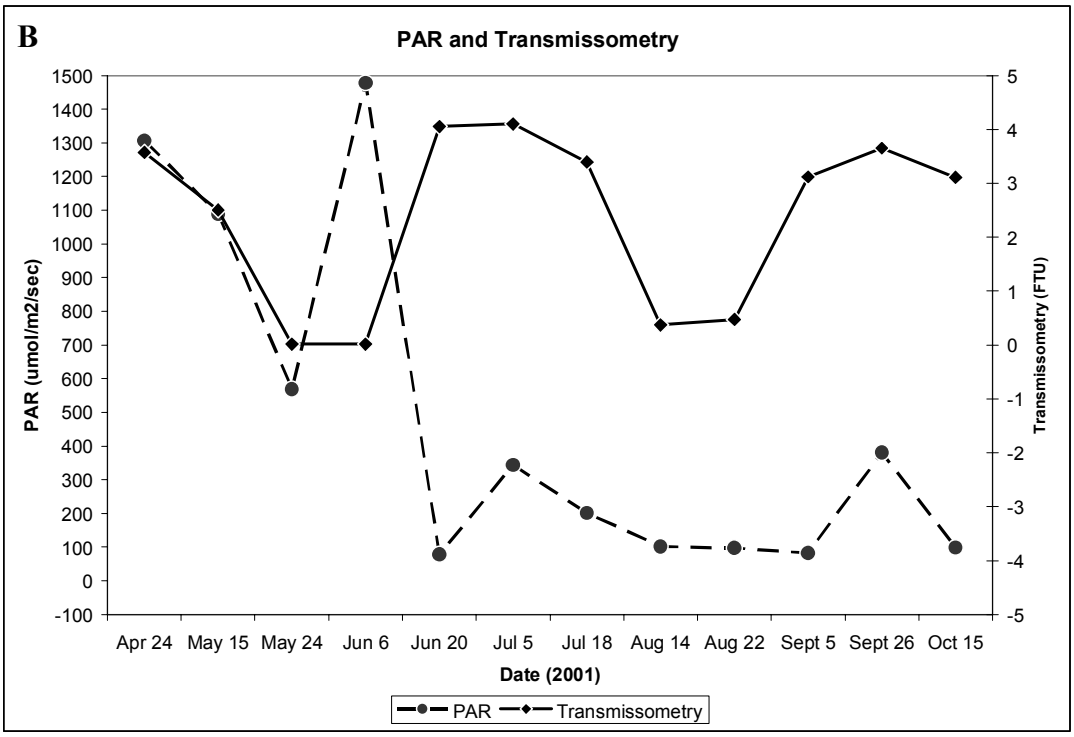


Figure 2.1. Environmental data collected from NOAA SeaKeys/C-Man station at Long Key for 2001. **A)** Salinity (ppt) and ocean temperature (°C). **B)** Photosynthetically active radiation (PAR, umol/m2/sec) and transmissometry (Formazine Turbidity Units, FTU).

Target Development

Total RNA was isolated from a 2 ml aliquot of each homogenized coral fragment following the manufacturer's protocol for TRIzol® (based on Chomezynski and Sacchi, 1987). RNA concentrations were estimated by ultraviolet absorbance at 260 nm and integrity of the ribosomal subunits was confirmed by electrophoresis on a 1% formaldehyde agarose gel. Replicate aliquots of up to 2 ug of total RNA from each sample collection were reverse transcribed using SuperScript™ II reverse transcriptase (Invitrogen™) and an oligo (dT) primer (Operon Biotechnologies, Inc.). During reverse transcription, DIG labeled dUTPs (digoxigenin-11-2'-deoxy-uridine-5'triphosophate, alkali-labile; Roche Diagnostics) were incorporated into the transcribed cDNA for subsequent detection using chemiluminescence. Specific conditions of the reverse transcription reaction are described in (Edge et al., 2005). For each sample collection, an aliquot of cDNA was added to a high sodium-dodecyl-sulfate (SDS) buffer (Roche Diagnostics) resulting in cDNA concentrations ranging from 30 – 50 ng ml-1. These DIG-labeled cDNA solutions represent the targets used with a coral array to assess differences in gene expression across collection date.

Expression Profiling

An experimentally designed coral gene array was used to evaluate differential gene expression in the field samples. ESTs on the array correspond to 32 different genes isolated from *Acropora cervicornis* and *Montastrea faveolata* (Morgan et al., 2001; Morgan and Snell, 2002; Edge et al., 2005). These gene fragments cover a range of functions including response to xenobiotic exposure and oxidative stress, maintenance of

cellular integrity and respiration, post-translational processing and apoptosis. The cDNAs representing each gene were spotted in triplicate onto each nylon array and samples were analyzed using three replicate arrays to estimate technical error. Edge et al. (2005) describe the development of the array and the preparation of probes on the array.

Hybridization

DIG-labeled targets were hybridized to the array in order to visualize probes expressed in the total RNA from colonies collected at each date. The hybridization protocol is described in Morgan et al., 2001 and Morgan and Snell, 2002. Hybridizations of samples from each date were performed three times using labeled cDNA from different colonies. Nylon membranes were used only once to ensure a consistent correlation between spot intensity and transcript concentration. Membranes exposed overnight to X-ray film produced dark spots corresponding to expressed transcripts in target cDNA. The level of expression between samples collected at each time point was compared. Array analysis to determine levels of probe expression is described in Edge et al., 2005.

Data Analysis

Global mean normalization was performed across all arrays. After background signals had been subtracted, all detectable signals were log 2 transformed. Statistical analyses (Univariate ANOVA, SPSS v.15) were performed on log 2 transformed data. Levene's Test of Equality of Error Variances was used to determine if all data groups had similar variances. If Levene's Test revealed significant differences in sample varianes, the Tahmane's T2 posthoc test was perform to determine which sample groups were

significantly different from each other. If the variances were similar, then Student-Neuman-Keuls test was used to determine if there were any significant subsets of sampling groups.

Clustering

Hierarchical clustering was used to develop a preliminary characterization of the genes and their corresponding expression profiles during the time course in this study. Mean values of log transformed expression data were calculated for each gene on every date. Hierarchical clustering was performed on these mean values in order to generate clusters of genes with similar expression patterns (Eisen et al., 1998).

Results

During this investigation, 12 of the 32 genes on the array were detected. Three independently prepared replicate membranes did not vary significantly in their expression signals ($P>0.05$,Univariate ANOVA, $F_{2,26}= 0.843$). Detectable expression signals ranged from a -3.84 to 6.48 log base 2 arbitrary units (au) above the background. Detectable signals with values less than 1 transformed into negative expression signals. Descriptive statistics of log transformed data indicated unequal variances for the 12 genes analyzed in this study (Levene's Test of Equality of Error Variances, $F_{415,629} = 4.892$; $P<0.5$). Since Univariate ANOVA is generally insensitive to heteroscedasticity, Tamhane's T2 posthoc test was applied (Tamhane, 1979).

Mean expression of individual genes

Of the 12 genes analyzed, the three ribosomal genes used in this study exhibited the highest average expression levels ranging from 4.94 to 6.31 au. Results of Tamhane's test revealed that the mean expression levels of these genes are not significantly different from each other ($P>0.05$). UC1 (uncharacterized 1) had the second highest expression signal ($x = 2.55$ au), especially on 22nd Aug and 5th Sept which were significantly different ($P<0.05$, Tamhane's T2 Test) from the levels expressed on 20th June and 18th July. Thioredoxin, uPAR and UC2 (uncharacterized 2) had the third highest mean expression signals ranging from 0.77 to 2.22 au. All of the remaining genes (TRAP, Mt, Ft, UC3, & PUcope17A) represented the fourth group which had the smallest mean expression signals ranging from 0.35 to 0.41 au.

Mean expression of all genes by date

One of the fundamental modes of characterizing gene expression profiles over a time course is to compare the collective signal intensities of all genes for each time period. There was a significant difference in the combined expression levels of all genes on different dates (Univariate ANOVA, $F_{120,237}$; $P<0.05$). Gene expression on 24th April was significantly different from all other dates except 18th July ($P<0.05$,Tamhane's T2 Test). Gene expression on 18th July was not significantly different from any of the dates examined in this study.

Clustering analysis

Hierarchial clustering (parameters: uncentered correlation with average linkage) was used to provide a basis for a preliminary characterization of the expression profiles for genes in this study. The analysis revealed that some of the genes could be grouped into clusters with varying degrees of similarity based upon their expression levels. The genes in an individual cluster were either directly linked, or closely joined by nodes, indicating a high degree of similarity based on the cluster analysis. This form of cluster analysis provides a platform for organizing the collective gene expression profiles of noisy population level data into discrete subgroups that can highlight the profile of each subgroup. The expression profile for the ribosomal genes produced cluster 1 which had significant similarities (Pearson's correlation coefficient = 0.99) and further illustrates how these three genes responded in a similar manner over the time course in this study (Figure 2.2). Hierarchal clustering grouped UC1, Thioredoxin, and uPAR into cluster 2 (Pearson's correlation coefficient = 0.88) (Figure 2.3A). Cluster 3 was composed of UC2, TRAP-D, and Ferritin (Pearson's correlation coefficient = 0.62) (Figure 2.4). The final, and fourth, cluster included the UC3 (uncharacterized 3) and PUcope17A (polyubiquitin) genes (Pearson's correlation coefficient = 0.22) (Figures 2.5 A and B). These two genes had the smallest expression signals and lowest correlation coefficients to any nearest neighbors. The MT gene did not cluster strongly with any other gene but was linked by two nodes to its nearest neighbor, which was cluster 3 (Pearson's correlation coefficient = 0.53) (Figure 2.6). Hierarchical clustering produces a final cluster that encompasses the entire group of genes. The calculated Pearson's correlation coefficient of this cluster (all

twelve genes) was -0.45. However, when the genes for cluster 4 were excluded from the collective cluster, the Pearson's correlation coefficient increased to 0.41.

Characterization of gene expression profiles by cluster groups

-Cluster 1

A comparison of the expression across dates of the ribosomal genes in cluster 1 using Tamhane's T2 test indicates significantly lower levels of expression on 24th April, 6th June, and 15th Oct compared to the three dates with the highest levels of expression (24th May, 22nd Aug, and 5th Sept) ($P<0.05$) (Figure 2.2). Temperatures on 24th April (24.9 °C) and 15th Oct (26.8 °C) represent two of the lowest temperatures recorded during this study (Figure 2.1A).

Figure 2.2. Cluster 1. Gene expression levels of three ribosomal proteins with a Pearson's correlation coefficient = 0.99. Sample collection dates in 2001 are indicated on the X-axis and log-transformed expression levels in arbitrary units on the Y-axis. Vertical lines indicate one standard deviation.

-Cluster 2

The genes in cluster 2 differed in their expression levels over time. The uncharacterized gene UC1 showed significantly higher levels of expression on 22nd Aug and 5th Sept as compared to 20th June and 18th July (P<0.05, Tamhane's T2 Test)(Figure 2.3B). Expression on 24th April appears lower in its expression level than either 20th June or 18th July, but is not statistically significant (P=0.06) because of greater variance (Figure 2.3B). Thioredoxin had elevated expression on 24th May compared to 24th April and 15th May (P<0.05, Tamhane's T2 Test) (Figure 2.3C). The uPAR gene was significantly lower in its level of expression on 14th Aug compared to 24th April, 20th June, 22nd Aug, 5th Sept, and 26th Sept. (P<0.05, Tamhane's T2 Test) (Figure 2.3D).

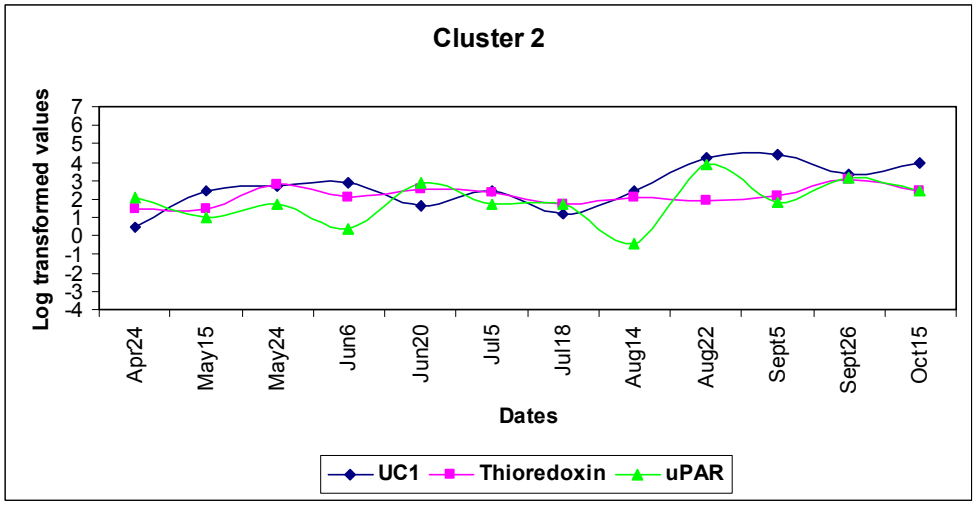


Figure 2.3A. Cluster 2. Pearson's correlation coefficient = 0.87. A negative value represents a log base 2 transformation of a signal that was <1.0 but still above the background.

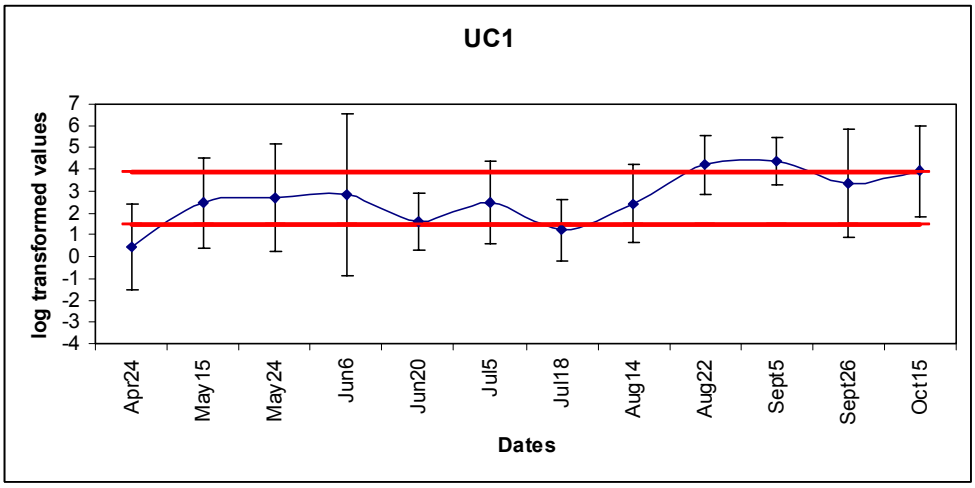


Figure 2.3B. Gene expression profile of uncharacterized (UC1). Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression levels on 22nd Aug and 5th Sept are significantly elevated compared to dates 20th June and 18th July.

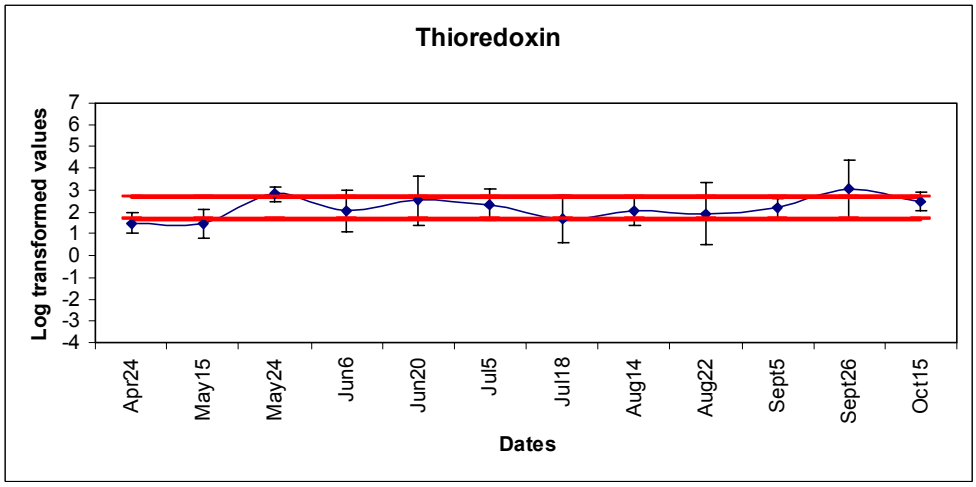


Figure 2.3C. Gene expression profile of Thioredoxin. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression levels on 24th April and 15th May are significantly lower than on 24th May.

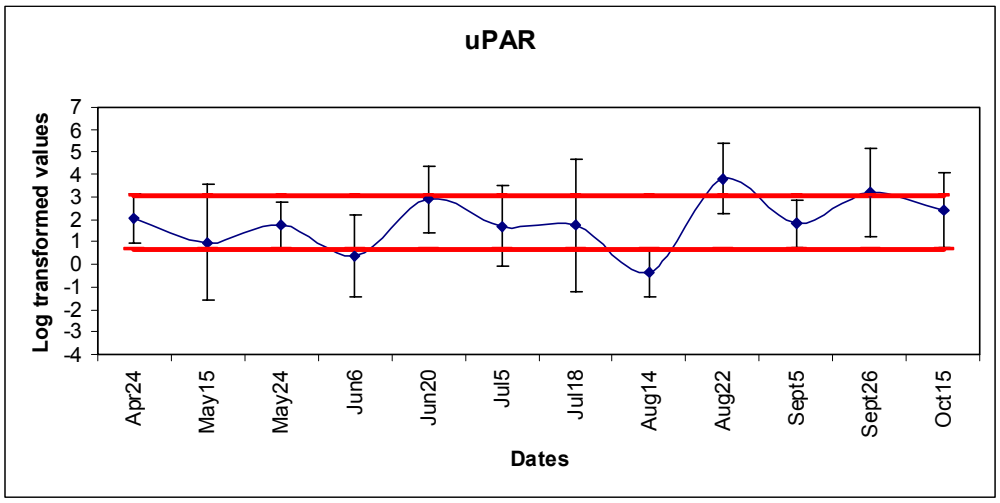


Figure 2.3D. Gene expression profile of uPAR. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression levels on 14th Aug were significantly lower than 24th Apr, 20th Jun, 22nd Aug, 5th Sept, and 26th Sept.

-Cluster 3

Signals on 18th July, 22nd Aug, and 26th Sept represent the greatest deviation from the mean level of expression for genes in cluster 3 (Figure 2.4A). Although the expression levels for the genes in this cluster are not identical, they share the common characteristic of elevated expression at some point in time spanning the dates of 22nd Aug through 26th Sept. PUsal showed significantly higher levels of expression on 22nd Aug and 26th Sept compared to 18th July ($P<0.05$, Tamhane's T2 Test) (Figure 2.4B). The TRAP gene was significantly elevated on 26th Sept compared to 6th June, 5th Sept, and 15th Oct ($P<0.05$, Tamhane's T2 Test) (Figure 2.4C). The Ferritin gene had an elevated expression level on 5th Sept compared to 15th May and 6th June, while expression on 26th Sept was significantly elevated compared to 15th May, 6th June, and 14th Aug ($P<0.05$, Tamhane's T2 Test) (Figure 2.4D).

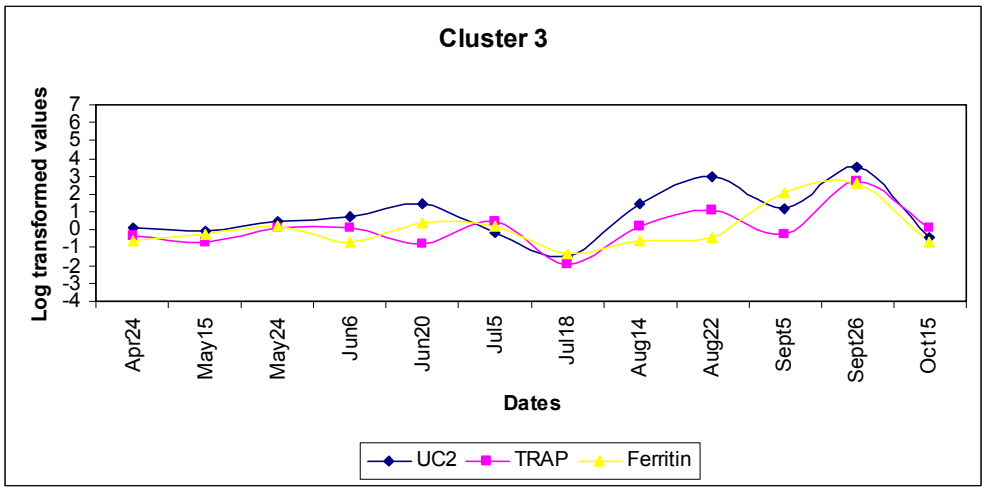


Figure 2.4A. Cluster 3. Pearson's correlation coefficient = 0.62. A negative value represents a log base 2 transformation of a signal that was <1.0 but still above the background.

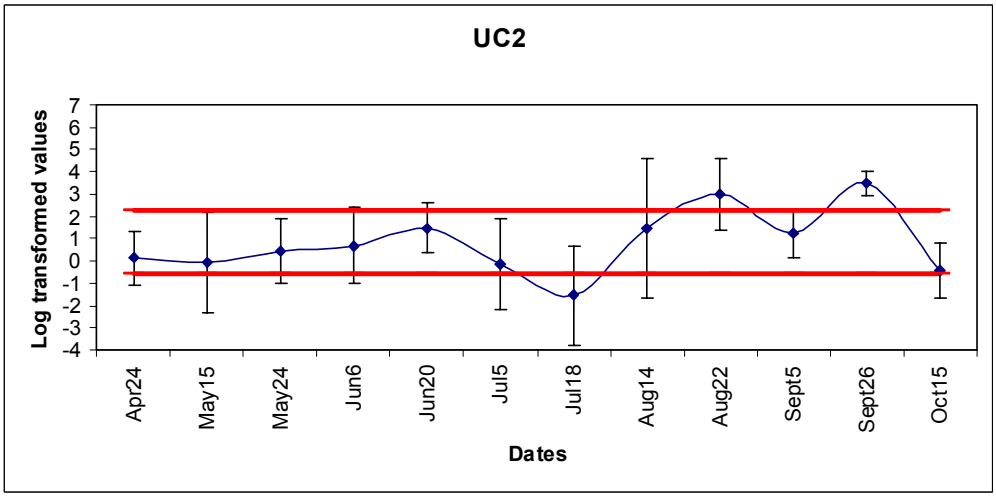


Figure 2.4B. Gene expression profile of uncharacterized (UC2). Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression levels on 22nd Aug and 26th Sept are significantly elevated compared to 18th July.

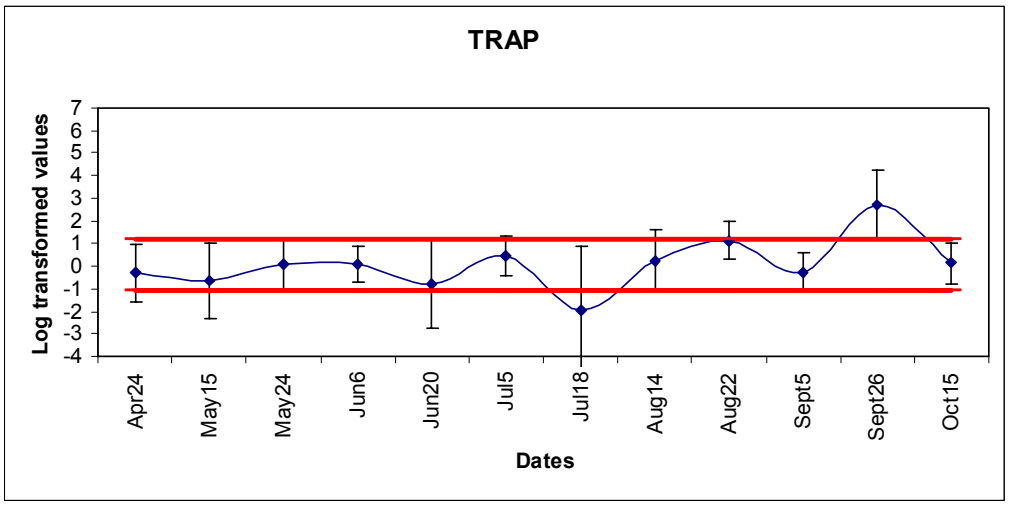


Figure 2.4C. Gene expression profile of TRAP. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression on 26th Sept is significantly elevated compared to 6th June, 5th Sept, and 15th Oct.

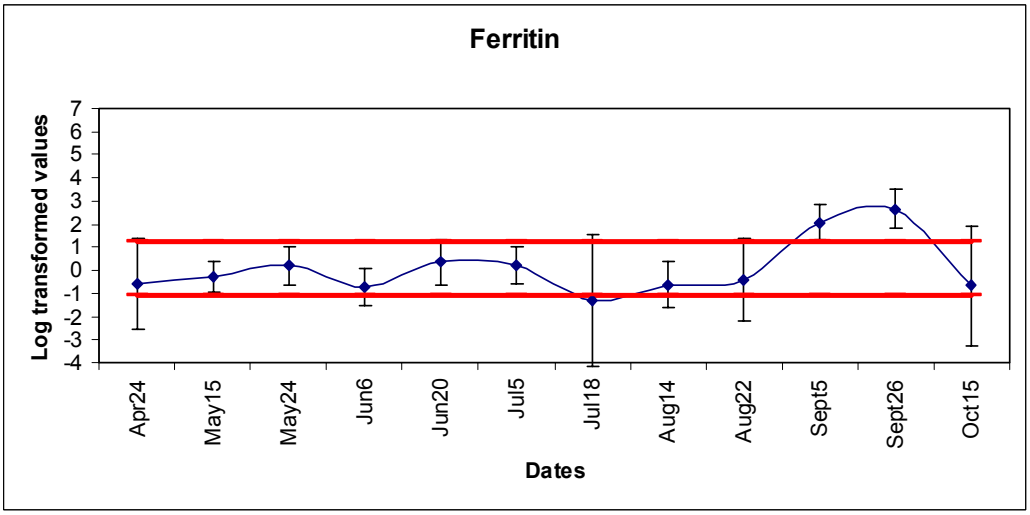


Figure 2.4D. Gene expression profile of Ferritin. Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Expression on 5th Sept is significantly elevated compared to 15th May and 6th June. Expression on 26th Sept is significantly elevated compared to 15th May, 6th June and 14th Aug.

-Cluster 4

Both genes (PUcope17A and UC3) exhibited no significant differences in their expression levels at any date during this study (Figure 2.5A). These two genes also exhibited the overall lowest levels of expression of any of the genes detected in this study (Figures 2.5B, 2.5C). The UC3 gene had no signal detected on 15th May (Figure 2.5B).

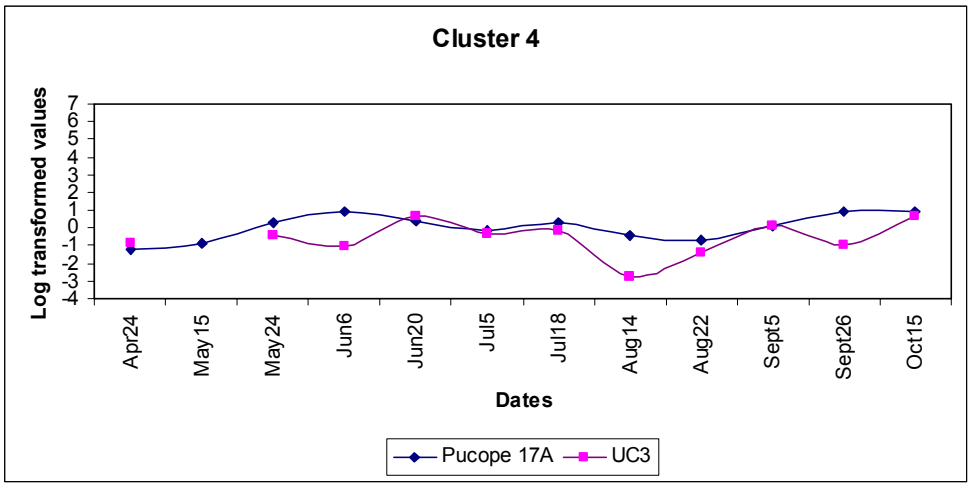


Figure 2.5A. A negative value represents a log base 2 transformation of a signal that was <1.0 but still above the background. No signal was detected for UC3 on 15th May since.

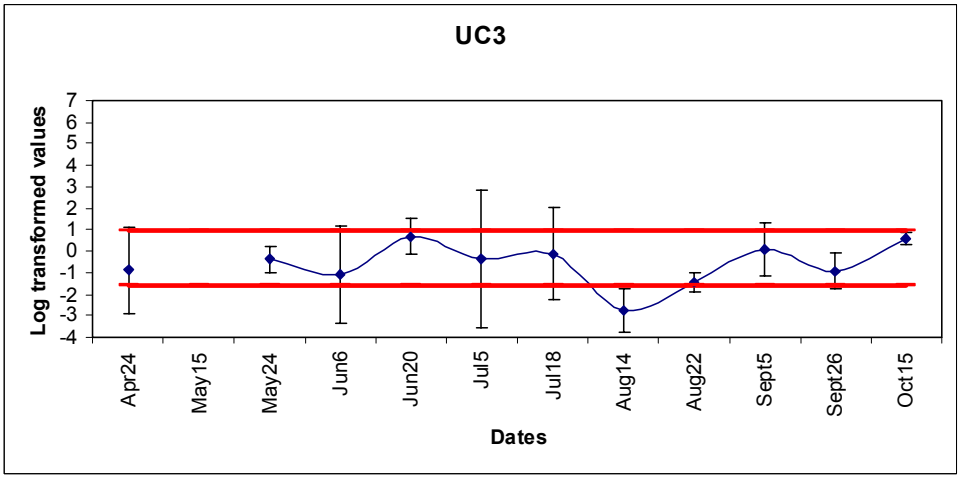


Figure 2.5B. Gene expression profile of uncharacterized gene (UC3). Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates. Note there was no signal detected on 15th May.

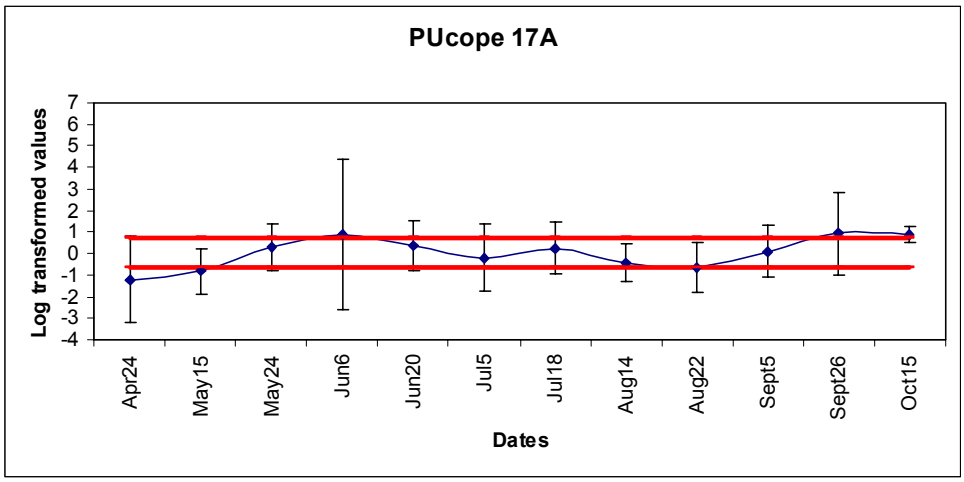


Figure 2.5C. Gene expression profile of polyubiquitin (PUcope17A). Error bars represent standard deviation for all signals from corresponding date. Solid horizontal trend lines represent 1 SD from the mean for all twelve dates.

-Non-clustered gene

The expression profile for MT (metallotionein) revealed that there were no significant differences in its expression levels during all the sampling dates (Figure 2.6). Levene's Test revealed similar variances between all samples, and Student-Neuman-Keuls test confirmed that all samples were not significantly different ($p>0.05$).

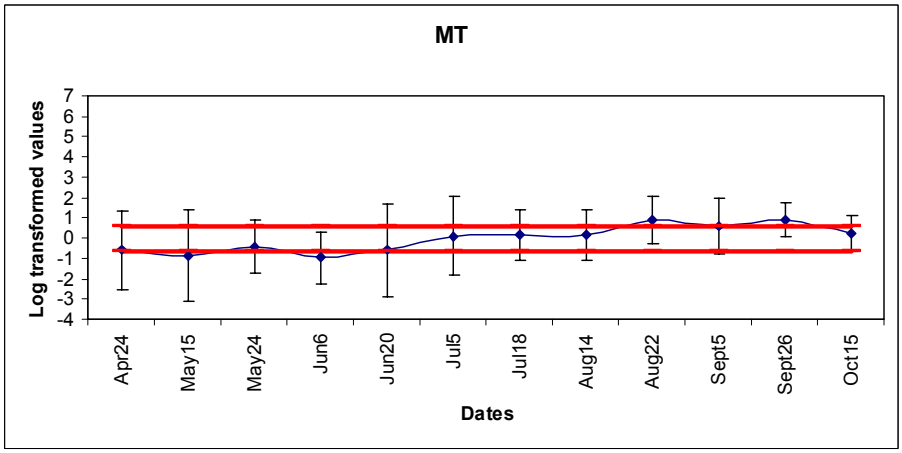


Figure 2.6. Gene expression profile of metallothionein (MT). A negative value represents a log base 2 transformation of a signal that was <1.0 but still above the background. This gene's nearest neighbors were Cluster 3.

Correlations with environmental metrics

The expression profiles were compared to the available environmental data. For each gene, the mean values of expression per date were compared to the mean values recorded for water temperature, salinity, transmissometry and PAR on the corresponding date. Pearson's coefficient correlations were calculated and summarized in Table 2.1. Genes in clusters 1 and 2 (ribosomal, UC1, Thioredoxin, and uPAR) demonstrated positive correlations with changes in temperature. These same genes also showed similar correlations with changes in the salinities over the time course of this investigation. In addition to their strong correlation to water temperature, two genes (Thio and uPAR) also exhibited positive correlations >0.80 with transmissometry. The genes in cluster 3 (TRAP, PUsal, and Ferritin) and cluster 4 (UC3, and PU cope 17A) did not exhibit strong correlation with any of the environmental metrics.

Table 2.1. Pearson's correlation coefficients for up-regulated genes and corresponding environmental metric.

Gene ID	Temperature	Salinity	Transmissometry	PAR
Ribos	0.98	0.97	0.79	0.59
UC1	0.92	0.90	0.71	0.47
UC3	0.02	0.02	0.11	-0.33
Mt	0.06	0.01	0.08	-0.70
UC2	0.54	0.51	0.32	0.21
PUcope17A	0.21	0.17	0.12	-0.12
Thio	0.97	0.97	0.83	0.61
Ferritin	0.29	0.26	0.32	-0.05
TRAP	0.14	0.11	0.05	0.03
uPAR	0.85	0.83	0.81	0.45

-Cluster 1

Ribosomal gene expression appears to correlate with changes in water temperature (Peason's correlation coefficient = 0.98). A 0.7°C change in temperature from 24th April to 15th May corresponds with a sharp rise in the expression of ribosomal genes during the same time period (Figures 2.1A and 2.2). There is a temporary drop in ribosomal expression during 6th June before stabilizing at an elevated rate from 20th June to 26th Sept. This corresponds to the highest recorded water temperatures during the collection period. Ribosomal expression and water temperature both drop on 15th Oct.

-Cluster 2

The expression profile of cluster 2 is different from cluster 1 (Figures 2.2A and 2.3A). The highest expression levels for the uncharacterized gene were evident on 22nd Aug and 5th Sept and lowest on 24th April, 20th June and 18th July. However, expression on 24th April was not significant from the highest levels detected on 22nd Aug and 5th Sept even though it was the lowest for any of the dates (P=0.06). This may be a function of the small sample size and the high variability associated with a population level study. The highest water temperatures were recorded on 14th Aug and 22nd Aug, while the highest expression levels of the uncharacterized gene occurred on 22nd Aug and 5th Sept. The expression level of this gene remains elevated on 15th Oct even though there is a 4°C drop in temperature between 26th Sept and 15th Oct (Figure 2.1A and 2.3B).

-Cluster 3

Cluster 3 has an expression profile that does not fluctuate from 24th April through 5th July (Figure 2.3A). On 18th July, there is a temporary drop in expression of all three genes (UC2, TRAP-D, and Ferritin) followed by elevated expression levels over the span of 22nd Aug to 26th Sept. The pattern of expression for these genes does not strongly correspond to changes in water temperature (Pearson's correlation coefficients, UC2 = 0.54; TRAP-D = 0.14; Ferritin = .29) (Table 2.1).

-Remaining genes

The uncharacterized gene (UC3) and the PUcope17A gene in cluster 4 show no significant correlation to any of the environmental metrics. However, the metallothionein gene (MT) shows a negative correlation to PAR measurements over the time course of this study (Pearson's correlations coefficient = -0.71) (Table 2.1).

Discussion

The profiles of genes observed in this study can be divided into two groups based on time period of expression. During spring and early summer, May into July, most of the genes show little deviation from their average level of expression with the exception of the ribosomal genes which show the greatest deviations in April and early June. However, in late summer, August and September several of the genes show larger oscillations around their average expression. These changes in expression across time reveal the natural variation of genes on this array within a coral population. In addition, detection of significant changes on certain dates above the average level of expression reveals the

capability of this cDNA array to detect fluctuations in gene expression within a natural population of coral that may not be associated with natural variation.

Differential Expression

During August and September a stimulus, or multiple stimuli, may be inducing gene expression. Several genes on the array exhibit different expression patterns during these sampling dates. The exact cause of these changes in gene expression is not currently evident; however hypotheses include environmental stimuli, such as elevated temperature, or physiological events, such as spawning. Three of the genes that fluctuate in August and September are uncharacterized, so there is no data on their molecular functions. Of these, UC1 correlates strongly with temperature and salinity data indicating response to environmental stimuli (Table 2.1). These results suggest hypotheses regarding which environmental stressors are having the greatest impact on field populations of coral at our collection sites. To confirm these hypotheses, tests in controlled conditions need to be quantitatively compared to the expression profiles observed in this field study.

Ferritin is elevated during both collection dates in September but does not correlate strongly with any of the recorded environmental data. Ferritin is involved in iron homeostasis, regulation of cell proliferation, and antioxidant defense (Aust, 1995; Orino et al., 2001; Kuo et al., 2004). Oxidative stress and exposure to ultraviolet radiation have been shown to increase ferritin expression (Cairo et al., 1995; Applegate et al., 1998; Pourzand et al., 1999; Tsuji et al., 2000). In marine organisms, oxidative stress is an

important part of the stress response and is associated with multiple environmental insults including thermal stress and exposure to ultraviolet radiation (Lesser, 2006).

Although uPAR fluctuates across all collection dates, its highest level of expression occurs in late August. The uPAR gene also exhibits relatively strong correlations with three of the four environmental parameters in this study (Table 2.1). Previous experiments have demonstrated elevated expression of uPAR in response to acute increases in temperature, salinity and UV exposure (Edge et al., 2005). This gene has multiple functions including signal transduction (Behrendt, 2004), regulation of proteolysis, cytokine activity and cellular adhesion (www.geneontology.org). It is found in several cell types, but is mostly expressed in tissue undergoing remodeling, since it is especially important in wound healing and matrix degradation (Behrendt, 2004). For example, human epithelial cells induce the expression of uPAR mRNA in response to UV light (Marschall et al., 1999). In addition, increased expression of uPAR has been detected in coral colonies from areas known to be experiencing elevated levels of sedimentation (Morgan et al., 2005).

Thioredoxin exhibits significantly different levels of expression during the first three sampling dates (Figure 2.2C). Thioredoxin is up-regulated in response to oxidative stress, UV exposure, hypoxia and acute exposure to elevated salinity; it is also involved in cell proliferation, growth and development, and signal transduction (Arner and Holmgren, 2000; Das and Das, 2000; Das and White, 2002; Edge et al., 2005). The significant differences in levels of expression of this gene coupled with its correlation to water

temperature and/or salinity early in the sampling dates suggest it may be a sensitive indicator of corals beginning to respond to seasonal changes in their environment.

Environmental Factors

The rapid rise in water temperature and its sustained elevation for several months is one possible stressor impacting corals during the sampling period. Undoubtedly, there could be a multitude of other environmental factors, such as sedimentation or UV, which may have influenced the observed expression profiles. It is noteworthy that 5 of the genes (UNC1, Thio, uPAR, Ft, Ribo) that show elevated expression on a few, if not all, collection dates are known to be induced by UV exposure (Marschall et al., 1999; Pourzand et al., 1999; Didier et al., 2001; Wang and VandeBerg, 2004; Edge et al., 2005) and are involved in the oxidative stress response (Cairo et al., 1995; Lee et al., 1999; Gasch et al., 2000; Didier et al., 2001; Walker et al., 2002). Ultraviolet radiation and the oxidative stress response are intimately connected. Exposure to ultraviolet (UV) radiation leads to protein damage, tissue inflammation, DNA damage and cell death either directly, or by generating reactive oxygen species (Miralles et al., 1998; Lesser et al., 2001). Organisms respond by up-regulating suites of genes that code for transcription factors, growth factors and proteases, which have been characterized in mammals as the UV response (Devary et al., 1992; Miralles et al., 1998).

It is important to point out that changes in mean expression of other genes on the array were not detected. For example, genes that have previously demonstrated sensitivity to pesticide and polycyclic aromatic hydrocarbon exposure were not expressed in corals

collected during this study. The lack of induction for these groups of genes suggests that, if present, these pollutants were below concentrations necessary to trigger stress gene expression. It is also possible that these genes may have responded to a brief pollutant exposure during the intervals between the sampling dates in this study. Alternatively, the coral population may have physiologically responded to a pollutant exposure without noticeable changes in gene expression.

Spawning

A recent article indicates that coral spawning in the Caribbean correlates with the average temperature during the month of spawning and that all corals release gametes at 28 – 30° C, except *M. annularis*, which release at 27 – 30°C (van Woesik et al., 2006). According to our data, these temperatures occurred during the months of June and July in 2001. However, historical data show that coral spawning of *Montastrea* species in the Florida Keys tends to occur in the months of August and September, which were the warmest of our collection (30.8 – 32.2 °C) (Szmant et al., 1997; Mendes and Woodley, 2002). Several genes on the array show elevated activity from mid-August through September and, for some, their expression patterns correlate with temperature (thioredoxin, ribosomal, uncharacterized, uPAR). Expression of these genes could be an indication of thermal stress; however it could also be a result of spawning activity, since it is unknown how coral spawning affects the expression of the genes used in this study. Although environmental stimuli of coral spawning have been described, the biochemical mechanisms that elicit physiological responses have not been characterized (Tarrant

2005). Further investigations are required to determine how spawning events affect the overall expression of genes in the coral transcriptome.

Natural Variation in Gene Expression

-Ribosomal Genes

The regulated expression of ribosomal genes is essential in maintaining homeostasis. Ribosomal genes are involved in protein biosynthesis, RNA binding, and transcription regulation among other crucial cellular functions. It is expected that they would be consistently expressed in organisms across time. However, ribosomal genes have also been shown to fluctuate in response to stress (Causton et al., 2001; Edge et al., 2005). In response to acute exposures to stress, ribosomal gene expression increases with elevated salinity and exposure to UV, but decreases with elevated temperature (Edge et al., 2005). This study is consistent with previous results and reveals that ribosomal expression increases as temperature increases, with a significant drop in expression in early June which represents the greatest change in temperature (+ 4.6°C) observed during any four sampling dates in this study. This drop in expression may be due to the rapid rate of increase in temperature, similar to the shock of acute exposure in Edge et al. (2005). As temperature peaks and begins to level off, ribosomal expression also levels off before dropping again in October as temperature decreases. Expression profiles are not available from November to March during this study. This information, along with temperature data for the same period, could clarify how ribosomal expression correlates with changes in temperature for coral populations at this site.

-Other Genes

Polyubiquitin is induced by DNA damage but also regulates protein degradation, location, activity and interaction with other proteins (Fornace et al., 1989; Nenoi, 1992; Schnell and Hicke, 2003; Varshavsky, 2006). Metallotionein is most well known for its role in heavy metal detoxification (Sato and Kondoh, 2002), but it also functions in a number of biochemical processes including gene expression, apoptosis, proliferation and differentiation (Kagi and Schaffer, 1988; Vallee, 1995; Palmiter, 1998; Davis and Cousins, 2000). The expression levels of metallothionein and polyubiquitin do not fluctuate significantly across collection dates. The observed profiles may be the natural variation in expression for these genes since they are consistently 'on'. However, the possibility that during this study corals were undergoing continuous exposure to a stressor or suite of stressors cannot be ruled out.

Biological relevance of expression profiles

The data generated in this study demonstrate how a small group of genes vary in their expression patterns over time in one coral population. At this preliminary stage, it is unknown whether any of these genes can be directly linked in their expression to any of the observed environmental parameters. Important clues about the biological relevance of the expression patterns observed in this study can be obtained when correlation coefficients are calculated for individual genes and various environmental metrics. Correlating relevant environmental data with observed gene expression profiles narrows the list of stimuli potentially responsible for altered gene expression and generates testable hypotheses for future studies.

The genes used in this study were initially isolated from corals exposed to laboratory induced stress conditions (Edge et al., 2005). Although all of these genes are known to function during the stress response, they also have roles in general cellular metabolism. Subsequently, acute exposures of coral to elevated temperature, salinity and UV provided a first glimpse at how these genes behave under controlled conditions in coral. In addition, some of the genes used in this study have demonstrated differential expression in corals from sites at varying distances to a point source of pollution and with different sedimentation profiles (Morgan et al., 2005). This is the first study to investigate the expression of these genes in a natural coral population across time and demonstrates the ability of our cDNA array to detect changes in gene expression.

Chapter 3

Profiling differential gene expression of corals along a transect of waters adjacent to the Bermuda municipal dump

Abstract

A coral cDNA array containing 32 genes was used to examine the gene expression profiles of coral populations located at four sites that varied with distance from a semi-submerged municipal dump in Castle Harbour, Bermuda (previously identified as a point source of anthropogenic stressors). Genes on the array represent transcripts induced under controlled laboratory conditions to a variety of stressors both natural (temperature, sediment, salinity, darkness) and xenobiotic (heavy metals, pesticides, PAH) in origin. The gene expression profiles produced revealed information about the types of stressors. Consistent with other studies undertaken in Castle Harbour, the coral cDNA array detected responses to heavy metals, sedimentation, as well as oxidative stress.

Introduction

Stress is the reduction of an organism's ability to maintain homeostasis as a result of cumulative modifications in multiple metabolic pathways (Gasch et al., 2000; Svensäter et al., 2000). The same stressor can elicit different responses depending on the organism's physiological status prior to exposure to the stressor(s). Factors such as the type of stressor, the period of exposure (both temporally and spatially), and previous physiological conditions can all influence the stress response. One means of determining whether corals are stressed is to compare the same response in different populations at the

same time. Unfortunately, population/community level studies suggest that most coral populations are experiencing at least some degree of stress, which has resulted in decreases in coral coverage and/or species diversity world wide (Jackson et al., 2001; Pandolfi et al., 2003). Working within the paradigm of "no corals are unstressed," valuable information can still be obtained by comparing different populations to see if each population is expressing the same stress response during the same time period. Genetic biomarkers expressed in one population that are up/down regulated relative to other populations can be valuable indicators of the relative position of each population along the continuum of healthy/stressed responses as the organisms continually attempt to maintain homeostasis.

Anthropogenic point sources: an application of ecotoxicogenomics

Identifying geographic locations as probable point sources of anthropogenic stressors offers ecotoxicologists the opportunity to investigate whether biomarkers responsive to anthropogenic stressors are capable of being detected along an exposure gradient from the point source. The study sites identified during the First International Ecotoxicology and Coral Health Workshop held in Bermuda in September 2003 (Owen et. al., 2005) provide a setting to investigate the effectiveness and sensitivity of a stress gene array in detecting a potential point source of xenobiotics, the island's semi-submerged municipal dump. In the developing field of ecotoxicogenomics, there are two main objectives: 1) to analyze the expression of several genes simultaneously in order to identify patterns of stress responses at the level of gene transcription and 2) to isolate and characterize the functionality of unknown genes which are differentially expressed in response to

stressors. Studying how expression patterns of stress-induced genes are altered in different environments allows diagnosis of where the positions of coral populations are along a healthy/stress continuum. With the development of stress gene arrays, possibilities now exist for comparing the expression of many genes simultaneously. An array composed of relatively few stress genes can provide valuable information regarding the ability of coral to respond under different environmental conditions, even if the actual stressor(s) remains unknown. The objective of this study was to use a 32 gene cDNA array to test corals from sites at varying distances from Bermuda's municipal dump in order to evaluate patterns of gene expression at each location.

Materials & Methods

Study sites

Four study sites were chosen, three were located along a transect line originating in proximity to a semi-submerged municipal dump within Castle Harbour (CH), a patch reef lagoon ecosystem located in north east of Bermuda (for details see Flood et. al., 2005; Quinn et. al., 2005). The site nearest the municipal dump was termed the Dump (site 1) and was located approximately 50 meters from this potential point source, the Annex (site 2) was located 960 meters from the dump, and Tuckers Town (site 3) was 2.73 km from the dump. North Rock, a site located 14.39 km from the dump on the edge of the outer fringing reef was chosen as a control site (site 4) since it is located in a region where open oceanic water exchange is high and where there is no direct circulation of water from Castle Harbour (Figure 3.1).

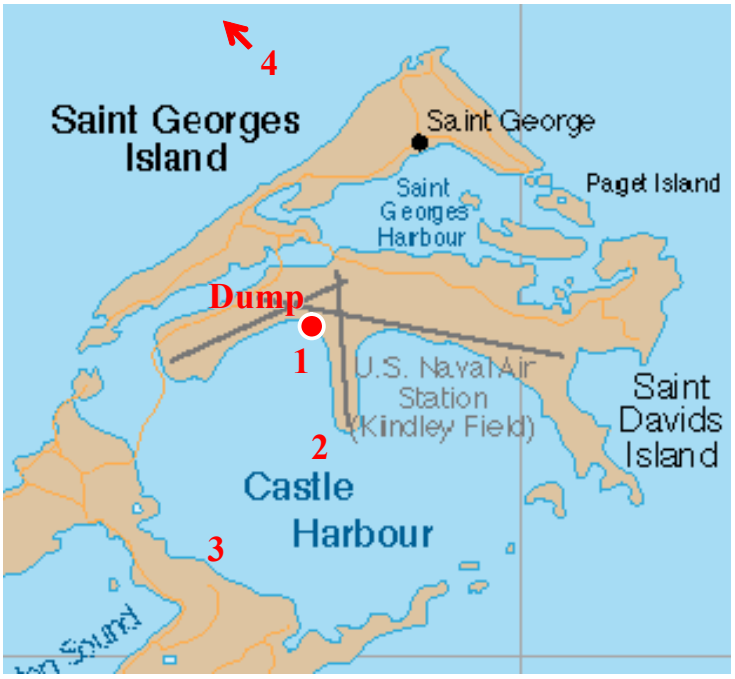


Figure 3.1. Map of Bermuda's Castle Harbour and the four sites in this study. The municipal dump in indicated by the red circle. Site 1 is 50 meters from the dump. Site 2 is 960 meters from the dump. Site 3 is 2.73 kilometers from the dump. Site 4, the control site, is located outside of the harbour and is 14.39 kilometers from the dump.

Coral sampling

Due to the limited coral diversity along the transect in Castle Harbour, *Diploria strigosa* was chosen as the coral to examine since it was one of the few species present at all sites. All samples were collected on November 18, 2003. Colonies sampled were taken from depths ranging from 2-4 meters within CH, while samples from North Rock were taken from depths ranging from 3-7 meters. Three centimeter square ($3cm^2$) cores from 3 different colonies at each site were collected. Coral tissues were brought onto the boat where they were immediately placed in liquid nitrogen in order to prevent degradation of nucleic acids. Frozen tissues were then shipped using a dry liquid nitrogen shipper to laboratories in Georgia for subsequent molecular analyses.

Extraction and manipulation of RNA

Excess calcium carbonate was removed with a chisel before total RNA was extracted from tissue by application of 4 ml of TRIzol® (Invitrogen, Carlsbad, CA, USA) to approximately 4-6 grams of coral skeleton plus tissue. Homogenization was accomplished by pulverizing samples in a mortar and pestle. After the homogenization in TRIzol®, the samples were divided into 1 ml aliquots of the respective treatment groups and the RNA extraction protocol was completed for each replicate/sample. Integrity and concentration of total RNA from holobiont (i.e. coral and symbiotic zooxanthellae) was confirmed by electrophoresis of an aliquot of each sample on a 1% formaldehyde agarose gel (Sambrook et al. 1989) and was compared to a standard (MS2, Roche Diagnostics, Indianapolis, IN, USA). Total RNA was further purified by DNase I digestion followed by phenol/$CHCl_3$ extraction (Message Clean®, GenHunter, Nashville, TN, USA).

Probe development

After total RNA purification, reverse transcriptions of mRNAs were performed using 1µg of total RNA, DIG-labeled UTPs (Roche Diagnostics, Indianapolis, IN, USA), oligo-dT primers, and reverse transcriptase (Superscript II, Invitrogen, USA) in 40 µl reactions. Concentrations of DIG-labeled cDNA produced were quantified by fluorimetry (DynaQuant, Amersham).

Detection of differential gene expression

DIG-labeled cDNA probes were used in Reverse Northern dot blot hybridizations to visualize the presence of target transcripts present in the total RNA samples from each

site. Probe solutions (~30ng/ml) from each site were hybridized to replicate membranes consisting of 32 genes that were previously isolated (Morgan et al., 2001; Morgan and Snell, 2002; Snell et al., 2003; Edge et al., 2005). Additional information about these 32 genes can be found in Edge et al. (2005). Each gene was spotted in triplicate for a total of 96 spots on the cDNA array. Immunodetection of probe hybridization was accomplished using anti-DIG antibodies conjugated to alkaline phosphatase (Roche Diagnostics, Indianapolis, IN, USA) to CSPD® substrate (Roche Diagnostics, Indianapolis, IN, USA). Chemiluminescent visualization was accomplished by exposing blots to autoradiographic film for 17 hrs.

Determining the expression profile for each population

Biologists commonly make comparisons based on fold-changes in expression. Such an approach was not used in this study since it assumes that each population is expressing the same suite of genes. In addition, our previous studies have demonstrated that the genes on our array exhibit various levels of stressor specificity; therefore to make fold-change comparisons infers that each population is being exposed to the same stressors at comparable concentrations. Densitometry of blots was performed using computer software Image J (National Institutes of Health, Bethesda, MD, USA). Measurements were recorded from replicate blots for every detectable gene on each membrane. Control genes were identified by homology to rRNA protein sequences (BLASTX 2.2.9, NCBI nr database). Background signals were quantified by measuring an area around each spot that represented twice the diameter measured within an individual spot. The intensity of each signal was initially determined by subtracting its adjacent background values.

Expression of an individual stress gene was determined by replicate blot signals for that gene on two different membranes.

In order to compare signal intensities of multiple spots on different membranes, all data were log transformed. This manipulation is considered a valid approach for analyzing data where the effects are believed to be multiplicative (Kerr et al., 2000). Analysis of variance (ANOVA) was then performed since it is capable of systematically estimating the normalization parameters on all relevant data (Kerr et al., 2000). Two different post hoc tests were used depending on the homogeneity of sample variances as identified by Levene's Test of Error Variances. If population variances were not significantly different from each other, then Student-Neuman-Keuls post hoc test was used. If population variances were significantly different, then Tamhane's T2 post hoc test was used.

Verification of expression

The purpose of this study was to apply the coral cDNA array as an initial screening tool to compare the expression profiles for selected populations. Other techniques with higher levels of sensitivity must subsequently be utilized to confirm expression of genes initially detected on the array. Northern dot blots were performed in manners previously described (Morgan et al., 2001; Morgan and Snell, 2002) to detect expression for genes particularly relevant to Castle Harbour.

Results

Fourteen of the 32 genes represented on the coral array were expressed at one or more locations within this study (Figure 3.2). All four locations showed similar patterns of expression for all ribosomal genes that were used as controls (Figure 3.3). Detectable log transformed expression signals ranged from a 0.75 to 1.77 arbitrary units (au) above the background. Statistical analysis of log transformed control gene expression data revealed similar levels of variance in the control genes expressed at each location ($P>0.05$, Levene's Test Equality of Error Variances). There were however significant differences (One-way ANOVA, $F_{3,92}=6.58$, $P<0.001$) in the mean signal intensities of the different control genes (Table 3.1). Collectively, expression levels for the control genes were significantly different depending on location. The Annex was significantly higher in expression of control genes compared to all other sites (Student-Newman-Keuls, $p<0.05$). Tuckers Town was also significantly higher than the Dump (Student-Newman-Keuls, $p<0.05$) but not significantly different from North Rock ($P>0.05$).

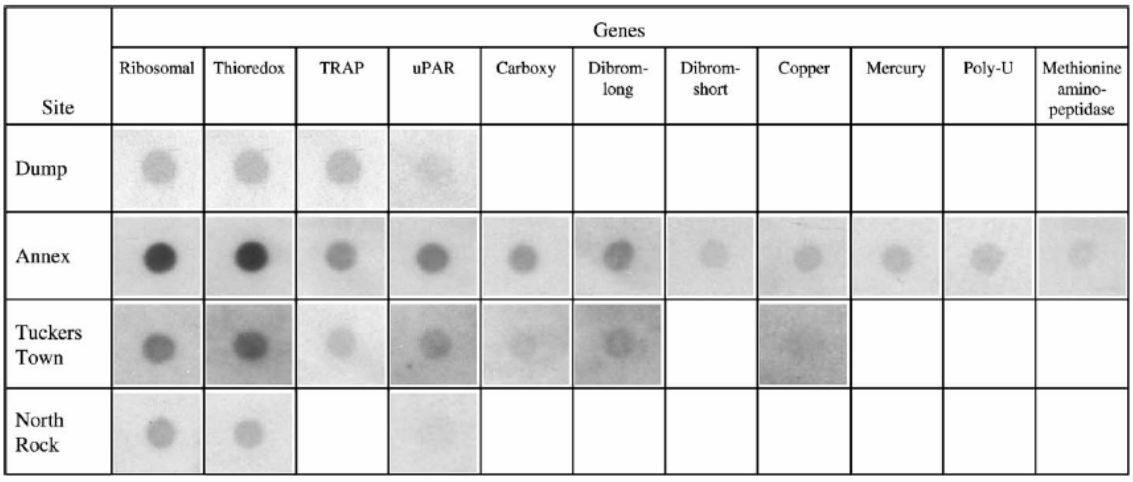


Figure 3.2. Representative dot blots for all genes expressed at each Bermuda study site. The ribosomal dot blot for each location is actually representative of four separate ribosomal genes.

Table 3.1. Spot intensities (x ± SE) of control genes on 8 membranes. Six replicates in each category were pooled to yield mean levels of expression. There were no significant differences of expression for any gene ($F_{3,92}$ = 1.3, P>0.05) within site, but there were significant differences for expression levels between sites ($F_{3,92}$ = 11.7, P<0.01). There was also a significant difference in the mean signal intensities of the different control genes (One-way ANOVA, $F_{3,92}$=6.58, P<0.001).

	Dump	Annex	Tuckers Town	North Rock	Gene across sites (P>0.05)
Ribosomal Gene 1	1.02 ± .16	1.86 ± .13	1.51 ± .11	1.44 ± .14	1.46 ± .07
Ribosomal Gene 2	.91 ± .15	1.82 ± .15	1.39 ± .16	1.27 ± .17	1.35 ± .07
Ribosomal Gene 3	.82 ± .13	1.71 ± .16	1.12 ± .11	.83 ± .23	1.12 ± .07
Ribosomal Gene 4	.89 ± .17	1.77 ± .22	1.32 ± .19	.85 ± .22	1.21 ± .09
Gene within site (P<0.01)	.91 ± .08	1.79 ± .08	1.34 ± .08	1.01 ± .08	

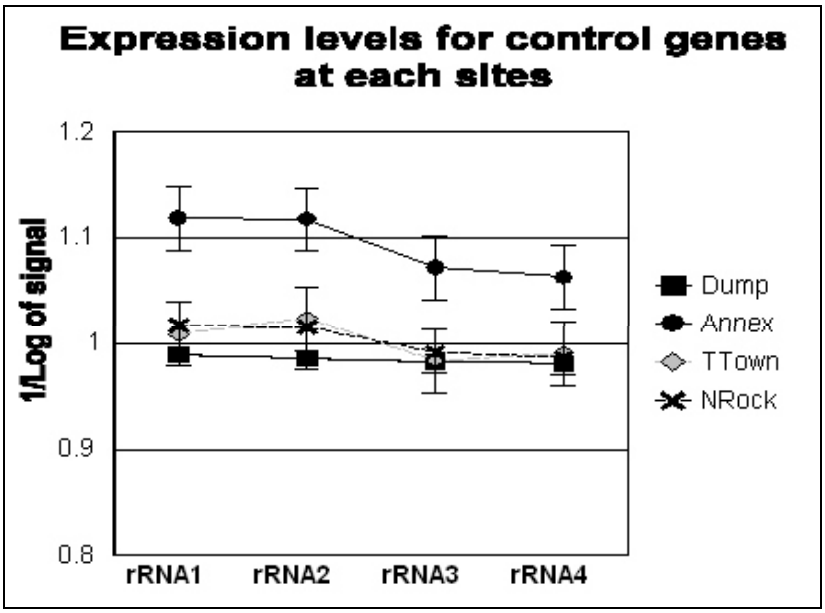
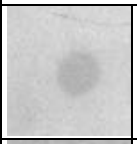
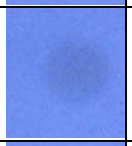
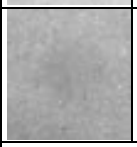
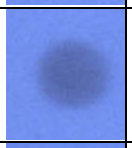


Figure 3.3. Expression of control genes at all four Bermudan study sites. Control genes expressed at Annex are significantly different in their levels of expression compared to the other sites (F = 11.7, P<0.01). Expression levels of all four control genes within a location do not differ significantly from each other (F = 1.3, P>0.5). All values have been normalized to the global average which equals the value of one on the y-axis.

Excluding control genes, coral from the Annex expressed the largest number of stress genes (10) followed by coral from Tuckers Town with six, the Dump with three, and North Rock with two (Figure 3.2). There were three stress genes that were expressed at all sites in Castle Harbour (Thioredoxin, TRAPD, and uPAR). Two stress genes (Thioredoxin and uPAR) were expressed at all four sites (Figure 3.2).

To confirm preliminary results, Northern dot blots were performed on a subset of genes originally expressed on the coral cDNA array. This subset represents transcripts that previously have demonstrated responsiveness to some of the same classes of stressors (e.g. metals) that have been previously reported within Castle Harbour (Burns et al., 1990). Results from Northern dot blots indicate that two genes (copper and uPAR) originally detected on the cDNA array were also detected on the Northern dot blots, however their expression profiles were not identical (Figure 3.4). The Northern dot blot for corals from Tuckers Town had the highest average expression signals for the uPAR transcript. Corals from all locations within Castle Harbour were significantly different in their expression signals of uPAR compared to corals from North Rock (Student-Newman-Keuls, $P<0.05$). The copper transcript was detected by Northern dot blots at every location in this study. Descriptive statistics of log transformed copper data indicated unequal variances (Levene's Test of Equality of Error Variances, $F_{3,8}=4.44$; $P<0.05$). Since univariate ANOVA is generally insensitive to heteroscedasticity, Tamhane's T2 posthoc test was applied (Tamhane, 1979). Results of Tamhane's test revealed that corals from all locations in this study exhibited similar expression levels of the copper transcript. The mercury and dibrom probes represented other signals detected

on the array that were of particular interest. Northern dot blots were unable to detect target transcripts from the mercury and dibrom probes.

Copper probe				
Location	Array blot	Array blot signal intensity	N. dot blot	N. dot blot signal intensity
Dump	ND			x = .75 ± .16 SE
Annex		x = 1.36 ± .09 SE		x=.98 ± .16 SE
Tuckers Town		x = .83 ± .11 SE		x = 1.31 ± .16 SE
North Rock	ND			x = 1.11 ± .16 SE

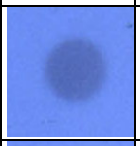
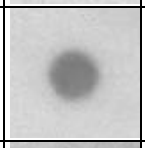
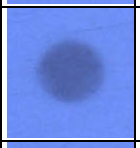
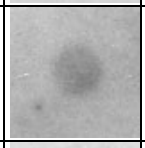
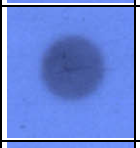
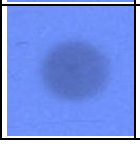
uPAR probe				
Location	Array blot	Array blot signal intensity	N. dot blot	N. dot blot signal intensity
Dump		x = .99 ± .01 SE		x = 1.56 ± .06 SE
Annex		x = 1.71 ± .1 SE		x = 1.59 ± .06 SE
Tuckers Town		x = 1.43 ± .1 SE		x = 1.81 ± .06 SE
North Rock		x = .82 ± .15 SE		x = 1.51 ± .06 SE

Figure 3.4. Signal comparisons from the cDNA array and Northern dot blots for the copper and uPAR genes. ND indicates a signal was not detected.

Discussion

Gene expression profiles

Results from the array suggest that each site is not expressing the same suite of genes (Figure 3.2) and the overall level of transcriptional activity varies between sites (Figure 3.3). Corals at the Annex appear to be most actively responding to stressor(s) by up-regulating 10 genes. Tuckers Town, located 1.5km from the dump, showed elevated expression of six stress genes on the array, while corals from the Dump site up-regulated expression of four stress genes. Our results suggests that in general, the coral populations in Castle Harbour have the greatest level of stress gene expression compared to corals collected at North Rock. At the North Rock control site, the array detected expression of the ribosomal genes and two stress-responsive genes (Thioredoxin and uPAR). Variability between sites could be due to differences in concentrations of toxicants. Previous studies using subsets of these gene probes have demonstrated that different concentrations of the same stressor can produce different levels of expression for individual transcript (Morgan et al., 2001; Morgan and Snell, 2002; Snell et al., 2003; Edge et al., 2005). The dump location exhibited a smaller number of induced stress genes which may be explained by differences in concentrations of the same stressor(s), or exposure to a different suite of stressors. Although every site in this study showed similar expression profiles of control genes, there were significant differences in the levels of transcriptional activity between sites. The reduced transcriptional activity by corals at the dump site is consistent with organisms experiencing a high degree of stress. It has been shown that under extreme stress, or extended duration of elevated stress, organisms may reduce transcriptional activity (Morel and Barouki, 1999; Gasch *et al*.,

2000). In Edge et al. (2005) coral gene expression decreased upon exposure to the highest temperature in a series of elevated, acute temperature exposures. Results from Northern dot blots demonstrated that expression of uPAR was significantly elevated at all three sites within Castle Harbour (CH) when compared to the control site at North Rock. Northern dot blots for uPAR exhibited patterns of expression consistent with blots detected on the cDNA array. Responses to heavy metals and pesticides proved more variable. The copper stress gene was initially detected on the array only for corals collected from the Annex and Tuckers Town sites, but Northern dot blots revealed its expression at comparable levels within all study sites. Northern dot blot analyses could not confirm the expression of the mercury or organophosphate transcripts which were initially detected on the array.

Even though preliminary results from cDNA array were more variable than Northern dot blots, collectively both assays help to identify subtle changes in physiology which suggest that corals at all sites are exposed to stressors capable of inducing expression of recognized stress genes. Thioredoxin (TRX) is known to be induced by a variety of oxidative stress conditions (Das and Das, 2000; Schallreuter and Wood, 2001; Das and White, 2002) and its expression at every location in this study suggests that all corals examined are experiencing this type of stress. This finding is consistent with oxidative stress responses detected in other marine invertebrates at these sites during the same time period (Quinn et al., 2005). Expression of the copper transcript by corals at all study locations suggests exposure to this heavy metal is ubiquitous around Bermuda. This conclusion is also supported by bioassays conducted on other marine invertebrates

(Quinn et al., 2005) as well as previous chemical analyses of ambient waters (Burns et al., 1990). Elevated expression levels of the uPAR transcript in Castle Harbour are consistent with genes expressed by corals under laboratory induced sedimentation stress (data unpublished) as well as other studies that have quantified elevated levels of sedimentation within Castle Harbour (Flood et al., 2005). Our results suggest that the coral populations in this study are expressing suites of stress genes consistent with these known stressors.

Probable sources of stressors

One plausible explanation for the pattern of stress gene expression observed in corals at the four sites would be the proximity to the dump in Castle Harbour. It is hypothesized that the dump, which is semi-submerged, is leaching out a complex mixture of chemicals into the water which is circulated throughout Castle Harbour. A previous investigation of water and sediments in Castle Harbour has identified significantly elevated levels of organic and trace metal contamination at a site adjacent to the dump compared to a control site within CH (Burns et al., 1990). However, a number of other plausible sources of stressors also exist. Bermuda's international airport is located near the municipal dump and thus represents a potential source of chemical pollutants. A non-point source of toxicants may also be coming from accumulated sediment within the harbour that can be periodically re-suspended by tides, currents, and hurricanes. Both recent (Linzey et al., 2003) and older studies (Burns et al., 1990) have demonstrated the presences of numerous organics (including pesticides) and heavy metals in the soils and/or sediments of Bermuda. Although no significant differences in the sedimentation

rates at various sites within Castle Harbour have been detected over the study period (Flood et al., 2005), that study reported overall higher sedimentation rates in Castle Harbour compared to control sites (outside CH). Flood et al. (2005) also reported flow rates in the area of Tuckers Town that are significantly higher than other sites in CH such as the Annex (Flood et al., 2005). The responses we have detected are consistent with corals responding to re-suspended and/or contaminated sediments.

Gene expression profiles reveal information about types of stressors

Our results suggest that corals in Castle Harbour have been exposed to various levels of a complex mixture of stressors capable of inducing the gene expression profiles observed. Two probable anthropogenic stressors are heavy metals and organics. Morgan and Snell (2002) demonstrated that corals can exhibit different gene profiles even when exposed to a small range of copper and mercury concentrations. Anti-fouling agents such as Irgarol 1051 are known to leach copper (Boxall et al., 2000) and studies have shown that Bermuda's coastal waters are contaminated with this biocide (Connelly et al., 2001). Copper has also been detected in sediments of CH (Burns et al., 1990). Another plausible explanation for expression of genes known to be induced by heavy metals could be a compound that has not previously been examined using our stress gene array. The anti-fouling agent Tributyltin (TBT) and its breakdown product (DBT) have been detected in the waters of Bermuda (Connelly et al., 2001), in spite of governmental regulations to restrict its use (Bermuda Government, 1989). Burns et al. (1990) detected elevated levels of zinc and lead in sediments in CH. Quinn et al. (2005) detected a suite of heavy metals in the soft tissues of bivalves (scallops) placed at each of these study sites over the period

October - December 2003. They also reported elevated levels of metallothionein-like proteins in the gills of scallops deployed at the Dump and Tuckers Town sites. In our study, expression of the metallothionein gene by corals was not detected on the cDNA array at any of the study sites. It should be noted that the copper, mercury, and metallothionein stress gene probes used in this study were developed by exposure of corals to a small number of stressors in a narrow range of concentrations (Morgan et al., 2001; Morgan and Snell, 2002). Future studies examining the expression of these genes will examine exposures to a wider variety of heavy metals including TBT, DBT, lead, cadmium, zinc, and nickel in order to clarify the specificity of their response. Experiments analyzing metal concentrations in coral tissues from the sites in this study will also be performed to confirm exposure and provide estimates of dose.

Expression of two dibrom probes in this study suggests exposure of the corals to some type of organic. PAHs have been detected in sediments from CH (Burns et al., 1990). The long-dibrom probe has previously been shown to be a more general response in corals that were exposed to organophosphates and/or elevated levels of PAHs, whereas the short-dibrom probe has been shown to exhibit a greater specificity to the concentration of an organophosphate stressor (Morgan and Snell, 2002). A previous study (Owen et al., 2002a) has reported seasonal inhibition of hemolymph acetylcholinesterase activity in bivalves deployed in the coastal waters of Bermuda, which may reflect exposure to organophosphates. That study, however, did not include Castle Harbour. Other types of organic residues have also been detected from soil samples in Bermuda, including DDE, DDT, Dicofol, Dieldrin, and PCBs (Linzey et al.,

2003). The gene expression profiles for corals at the Annex and Tuckers Town sites are consistent with previous studies (Morgan and Snell, 2002; Owen et al., 2002a) that have demonstrated responses to exposures of organophosphates in tropical marine invertebrates. Terrigenous sources of pesticides (Linzey et al., 2003) coupled with Castle Harbour sedimentation data (Flood et al., 2005) suggests that other sources and/or locations in CH besides the dump could be impacting the coral populations in this study. Since Northern dot blots could not confirm expression of these organophosphate probes, future studies should seek to further characterize the specificity of these probes in corals exposed to diverse classes of organics.

The three stress genes expressed at all locations within CH have previously demonstrated photo-inducible expression in other organisms. Thioredoxin (TRX) is capable of sequestering reactive oxygen species (Das and Das, 2000; Das and White, 2002) and has also been identified as a component of an environmental stress response in model organisms (Gasch et al., 2000). Translocon-associated protein delta (TRAP-γ) is a subunit of a transmembrane protein complex located at the site where nascent secretory proteins enter the endoplasmic reticulum (Hartmann et al., 1993; Hothuis et al., 1995). Expression of TRAP-γ has been linked to UVB exposure (Wang and VandeBerg, 2004) and has recently been identified as part of an ubiquitinated protein complex that forms cytotoxic aggregates (Miyazaki et al., 2004). Urokinase-Type Plasminogen Activator Receptor (uPAR) has been induced by exposure to UVB (Marschall et al., 1999) and is known to be actively involved in tissue remodeling such as wound healing by proteolytic degradation of extracellular matrices (Ploug 2003). It would be tempting to attribute the

expression of these genes by corals in CH to photon exposures. However, these genes are known to be induced under diverse conditions. In fact, we originally isolated TRX and TRAP-γ from laboratory exposures of coral to heavy sediment load. Although the uPAR gene was originally isolated from corals kept in the dark for two days, we have also detected its expression under laboratory induced sedimentation stress (data unpublished). From the responses we have detected, expression of all three genes can be linked to exposure to elevated sedimentation. Coral species such as *D. strigosa* are known for their ability to thrive in environments of high sedimentation (Bak and Elgershuzien, 1976) and there is evidence that coral community structure in Castle Harbour has shifted towards this sediment-tolerant species (Flood et al., 2005). Expression of uPAR in this study is consistent with a coral tissue remodeling response in an environment of elevated sedimentation. Previous studies on correlating depth profiles and oxidative stress have concluded that coral populations at greater depths exhibit increased oxidative stress responses during periods of thermal stress (Downs et. al., 2002). We cannot exclude the possibility that these genes (uPAR, Thioredoxin, TRAP-γ) could be induced by different stressors at different locations. However, it seems unlikely their expression is correlated to differences in depth at these high latitude study sites in November.

Effects of multiple stressors

Chemical analyses and toxicity tests performed on soils, sediments, and waters in Bermuda indicate that multiple anthropogenic stressors are ubiquitous (Burns et al., 1990; Connelly et al., 2001; Owen et al., 2002b; Linzey et al., 2003). Previous studies have

demonstrated that different stressors may elicit the same response. For example, elevated temperature (Jokiel and Coles, 1990; Gates et al., 1992; Fitt et al., 1993; Winter et al., 1998), exposures to heavy metals (Harland and Brown, 1989; Jones, 1997), UV irradiance (Gleason, 1993; Lesser, 1990; Lesser, 1997) or cyanide (Jones and Steven, 1997) have all been shown to induce coral bleaching. Collectively, these studies illustrate how coral responses to diverse stressors converge into common biochemical pathways. Some of the genes used in this study may best be characterized as general stress responses since they are known to be induced by multiple stressors. The stress genes detected in this study have one common characteristic, they were all originally detected/isolated from corals exhibiting up-regulated responses to stress. Molecular responses represent individual components of a more comprehensive physiological response and an attempt by corals to maintain and/or return to a stable homeostatic condition. The expression profiles for corals from all sites within CH indicate that corals are making site-specific physiological adjustments that are clearly different from each other as well as being different from the corals at North Rock.

Temporal variability in stressor exposure

Without constant monitoring of Castle Harbour, intermittent exposures of anthropogenic stressors may go undetected. The gene expression profiles generated in this study represent a suite of responses measured at one point in time. Repeated sampling for extended periods of time may lead to different trends or may reinforce observed responses. Studies have shown that elevated levels of some anthropogenic stressors in Bermuda occur seasonally with, for example, increased boating activities (Owen et al.,

2002b). Variability in exposure can also be due to sediment re-suspension events that can occur regularly with tidal changes, seasonal rainfall, or as the result of intermittent natural events such as hurricanes (Bermuda experienced a category 3 hurricane in early September 2003).

Tolerance

Development of tolerance associated with chronic stressor exposures may also explain the observed gene expression profiles. Corals are known to exhibit some metal tolerance (Harland and Brown, 1989) which could influence the abundance of specific transcripts. If the genes detected in this study are associated with a tolerance response, then dot blot signals could potentially be influenced by previous exposures. Another possibility is that the heavy metal concentration at the dump site exceeds the level necessary to induce expression of the genes. Metal concentration has previously been shown to be a key element in the expression of a coral metallothionein gene (Snell et al., 2003). The genes used in this study were originally isolated from corals that were exposed to heavy metals for only four hours (Morgan et al., 2001; Morgan and Snell, 2002; Snell et al., 2003). Without knowing the dose-response characteristics of each of the genes used in this study, it is plausible that exposure length and/or concentration may influence expression of the target transcripts.

Potential sources of variability

-Membrane effect

Statistical analysis of control gene expression suggest that observed differences between

locations reflect true population differences and were not artifacts of membrane preparation. Each location expressed all control genes in similar patterns with comparable variances. Likewise, there was no significant difference among the blotted replicates for an individual control gene ($F_{3,92} = 1.3$, $P>0.05$). Analysis of background signals indicated that all membranes had similar levels of background ($P>0.05$, data unpublished). These statistical analyses affirm that individual arrays were produced in a consistent manner. However, another element of membrane variability could exist in the form of independent PCR amplifications of replicate blots of rare stress genes. If each independent PCR reaction did not amplify the same quantity of product, then individual replicate blots may not contain the exact same amount of amplified target. Detectable thresholds for rare genes will be critically important. Most of the genes detected on the array gave signals in all three replicate blots. However, two genes (copper and uPAR) had less than three signals from the replicates and yet the results from Northern dot blots revealed that these genes were expressed (Figure 3.4). The quantity of cDNA on replicate blots on the array was originally quantified by comparisons to DNA mass ladders using gel electrophoresis (Edge et al., 2005). Future versions of the coral array should quantify the amounts of blotted cDNAs by more sensitive techniques such as fluorimetry.

-Efficiency of probe labeling and/or annealing

The same amount of total RNA (1µg) was used in the reverse transcription (RT) reaction for each population. The RT reactions for all four populations were performed at the same time to ensure uniformity of reagents and conditions. Each DIG-labeled probe

produced was then quantified by fluorimetry in order to ensure the probe concentration (29ng/ml) would be the same for the hybridization solution of each population. If we assume that the efficiency of the DIG-labeling reactions are not altered by the relative abundance of individual mRNAs, then signals expressed on the array should accurately represent relative differences in populations. In Figure 3.2, we see that corals at each location are exhibiting similar patterns of transcriptional activity for the control genes even though the levels of activity are elevated for those at the Annex and Tuckers Town sites. Figure 3.2 by itself would suggest that the efficiency of probe labeling may be different at the Annex compared to the other sites. When we compare the three stress genes commonly expressed within CH, we see there are no significant differences between either the genes or the locations (Two-way ANOVA, $P>0.05$). One would expect that elevated expression signals for the control genes observed at the Annex would also be exhibited in all other signals expressed at that location as well if probe labeling efficiency were an issue (see Figure 3.3). By comparison, Northern dot blots detected expression signals of selected genes within all populations. Expression profiles of uPAR on both the cDNA array and Northern dot blots, coupled with statistical analyses, suggest observed differences in expression patterns may represent real population differences between sites within Castle Harbour and the control site of North Rock. Techniques with greater resolution (i.e. Northern dot blots) are capable of detecting expression patterns for selected genes that may indeed differ from the initial results obtained with the cDNA array. It should be noted that it is not only more labor intensive but also more expensive to prepare 32 individual DIG-labeled probe reactions compared to one DIG-labeled reverse-transcription reaction. As a preliminary screening tool of populations, the cDNA

array can generate new hypotheses to be investigated and should be complemented by other techniques. Discrepancies between the cDNA array and Northern dot blots suggest there were differences in the sensitivity of responses detected by each technique even though hybridization conditions were identical for all membranes (both cDNA arrays and Northern dot blots). Genes not detected on the array at some sites, were subsequently detected at those same sites using Northern dot blots. These differences are best characterized as false-negative results. Studies have shown that false-negative results are more common (compared to false-positives) in micro-arrays (Peplies et al., 2003). Investigations looking at strategies for optimizing micro-array hybridizations have determined that secondary structures of target molecules can reduce the accessibility of probe binding sites (Peplies et al., 2003). To reduce the potential influence of interfering secondary structure, future investigations/applications using the coral cDNA array should optimize hybridization conditions. Studies have demonstrated that reducing the formamide concentration in the hybridization buffer and elevating the hybridization temperature to 46°C will decrease the occurrence of false-negative results (Peplies et al., 2003). To consistently detect rarer transcripts and thus help reduce the variability observed in this study, future applications of the coral cDNA array should include mRNA enrichment in the probe labeling RT reactions. Since microgram quantities of mRNA are difficult to obtain, techniques capable of amplifying small amounts of mRNA have been developed that don't significantly distort the proportions of transcripts expressed (Baugh et al., 2001).

Probe specificity in different species

All genes in these analyses were originally detected and isolated from species other than *Diploria strigosa* exposed to various laboratory stressors (Morgan et al., 2001; Morgan and Snell, 2002; Snell et al., 2003; Edge et al., 2005). While some coral genes appear to be highly conserved (van Oppen et al., 1999), expression studies from other organisms suggest that a species may contain a gene which lacks a homolog in closely related species (Rubin et al., 2000). In this study, it appears from detectable expression signals that 14 of the genes examined do have significant homology between species (see Fig 3.2). Other Cnidarian genomic studies have demonstrated that mitochondrial genomes of numerous species exhibit low genetic diversities (Shearer et al., 2002). Detection of target transcripts in other coral species broadens the applicability of our microarray in gene expression profiling. Our results demonstrate that molecular probes isolated from *Acorpora cervicornis* and *Montastrea faevolata* can be applied to other coral species such as *Diploria strigosa*. The data in this study represents baseline information on inter-species specificity. The possibility exists that expression signals detected in *D. strigosa* are not representative of the target transcripts in *A. cervicornis* or *M. faevolata* even though hybridization washes were performed under high stringency conditions. Detectable results from "cross-hybridizations" between different species provides information about similar sequences, however sequence similarities do not automatically correlate with similar function (Milkos and Maleszka, 2001).

Even though 14 genes did give a signal, there were an additional 18 genes on the array that did not give a detectable signal. A number of plausible explanations exists for the lack of expression signals. First of all, studies from model genomic organisms have

demonstrated that only subsets of known stress genes are expressed at any one time (Adams et al., 2000; Gasch et al., 2000; Hill et al., 2000). It therefore seems highly improbable that the coral cDNA array is entirely composed of stress genes that will all be expressed under the environmental conditions represented in this study. Secondly, *D. strigosa* genes may be highly homologous to our *Acropora* and *Montastrea* probes but these genes simply were not expressed in this study. Thirdly, *D. strigosa* may have expressed these genes but sequence similarities were insufficient to withstand the high stringency washes during hybridizations. Lastly, these genes may have been expressed below the level of detection for the techniques used in this study. Differences in detectable levels of expression can also account for the discrepancies between the expression signals initially detected on the array and the subsequent expression signals detected by Northern dot blots. Coupling Real-Time PCR with additional gene sequence information from *D. strigosa* should allow future studies to discern which explanation is most appropriate.

It is also possible that genes originally up-regulated in *Acropora* or *Montastrea* are constitutively expressed in other coral species such as *D. strigosa*. This option, however seems highly unlikely since constitutive expression should increase the probability of detecting signals for the majority of genes (if not all genes) on the array. Future genomic studies will be needed to determine whether the *D. strigosa* signals detected on the array represent expression of orthologous genes or well-conserved paralogs (related but perform different function) of *Acropora* and *Monstastrea* genes.

Conclusions

Gene expression profiling represents an excellent tool for comparing and detecting subtle differences in the health/physiology of coral populations. Our gene array detected responses to heavy metals, sedimentation, and oxidative stress at the locations sampled in this study. These findings are consistent with the pollutants/stressors reported in other studies as well as our previous laboratory induced stress exposures. Future studies will be performed to confirm these results by testing contaminant profiles in Castle Harbour water and sediments and analyzing chemical uptake by coral tissue. In addition, corals will be exposed to dump effluent in controlled laboratory studies and the resulting gene expression patterns measured. These expression patterns will be quantitatively compared to expression patterns observed in this study to confirm or reject the hypothesis that exposure to chemicals leaching from the dump induces the detected stress genes in coral. Expression of the thioredoxin, copper, and uPAR genes at the control location (North Rock), some distance from mainland Bermuda, also reaffirmed previous conclusions that all coral populations are experiencing some level of stress. Although previous studies have reported high concentrations of various anthropogenic stressors within and surrounding Castle Harbour, there still are corals surviving at these locations. This suggests that the gene probes on our array detect sub-lethal responses to stress which reaffirms the concept that gene expression profiling provides insight into subtle alterations in physiology employed by organisms to maintain homeostasis.

Chapter 4

Application of a focused microarray to quantify coral stress on South Florida reefs

Abstract

Coral communities are increasingly impacted by a variety of natural and anthropogenic stressors that act on local or global scales. Based on the type of stressor and scale of impact, corals exhibit different responses. A better understanding is needed regarding coral responses to the cumulative effects of stressors to minimize the impacts and aid in the implementation of effective management strategies. Gene expression profiles can be used to diagnose which stressors are impacting coral populations in the field. In this study, a focused coral gene microarray was used to detect changes in gene expression patterns of coral in South Florida associated with changing environmental conditions. It demonstrates that valuable information can be obtained by comparing coral gene expression profiles in the field to determine if populations are experiencing similar stress over time. Such comparisons between populations can aid resource managers in decision making by prioritizing reefs with the greatest threat of serious impact or decline.

Introduction

Coral communities are increasingly impacted by a variety of natural and anthropogenic stressors. As a result, coral reefs have shown a steady decline in diversity and abundance worldwide (Hoegh-Guldberg, 1999; Pandolfi et al., 2003; Wilkinson, 2004). The synergistic impact of global and local stressors is likely to cause further degradation of

reef systems through irreversible change (Buddemeier et al., 2004; Wilkinson, 2004). A better understanding is needed regarding how corals experiencing local stress, such as pollution or effluent exposure, respond to the cumulative effects of global stress, such as elevated temperatures associated with climate change. Altered land use, urban development, chemical pollution and improper waste disposal contribute to the decline of local coral reef communities through point source and non-point source impacts. To mitigate detrimental effects, reef stressors need to be prioritized by greatest impact so that effective management can be implemented. Corals will have improved prospects for survival if the impacts of local stressors can be minimized during this period of global climate change.

Molecular technology can be used to diagnose coral stress before obvious physiological changes like bleaching occur. Gene expression profiling identifies the temporal and spatial regulation of genes expressed by an organism in response to environmental perturbations (Causton et al., 2001; Edge et al., 2005). The expression of specific genes is altered to protect cellular structures, repair damage, and maintain normal cellular functions. Differential gene expression can identify gene function, elucidate the mechanisms behind a biological response and produce a snapshot of cellular machinery in action (Snape et al., 2004). Recently, gene expression profiling with cDNA arrays has been used to diagnose and quantify the impact of stressors on coral in the lab as well as the field (Edge et al., 2005; Morgan et al., 2005).

Advancements in microarray technology, including increased flexibility, customized arrays and lower costs have permitted the use of this technology in the study of non-

model organisms (Gershon, 2004). In addition, with the increasing availability of Cnidarian sequence information it is now feasible to incorporate hundreds of coral genes onto an array and monitor their expression in a single experiment (Edge et al., 2005; McKillen et al., 2005). In the first study to use a focused Cnidarian microarray to investigate coral stress in the field, *Monstastraea cavernosa* from different sites along the South Florida coast were monitored for over a year. Exposure to site-specific stress, related to point sources, seasonal influences, and global stressors were compared based on altered gene expression profiles.

Materials and Methods

Site Selection and Sample Collection

Five study sites at depths of either 10 or 20 meters were selected along the southeast Florida coast and marked with underwater buoys. The sites were offshore of the Port of Miami inlet and spanned a distance of approximately 15 kilometers. Sites one (20 m depth) and two (10 m) were 5 to 6 kilometers south of the inlet, while site three (10 m) was approximately 6 kilometers north and sites four (10 m) and five (20 m) were approximately 20 kilometers north of the inlet (Figure 4.1). Ocean temperature data was recorded daily using an Acoustic Doppler Current Profiler (ADCP) situated near South Site 1 (20 m) a meter off the bottom (Figure 4.2a). Precipitation and storm data was acquired from the NOAA National Data Center. Precipitation was recorded hourly by the Miami, Florida Dade County station (COOPID 85663) (Figure 4.2b).

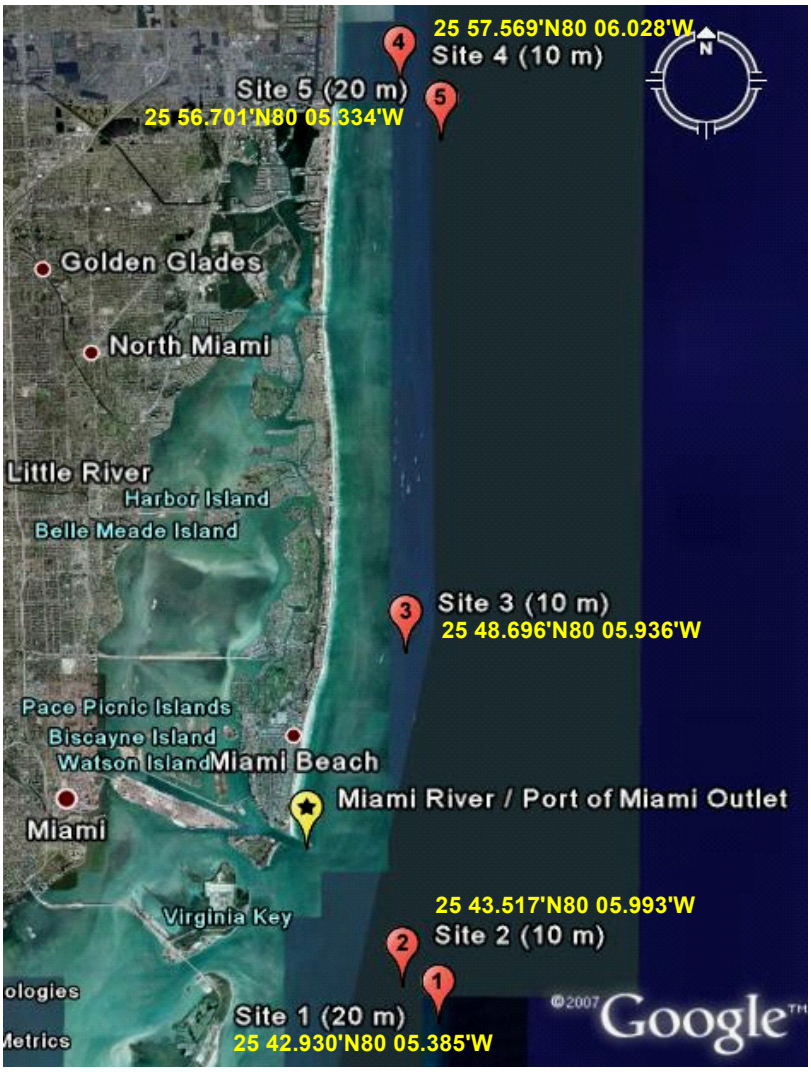


Figure 4.1. Map of collections sites produced using GoogleTM Earth. Depths, in meters, are listed after the site in parentheses and GPS coordinates are noted.

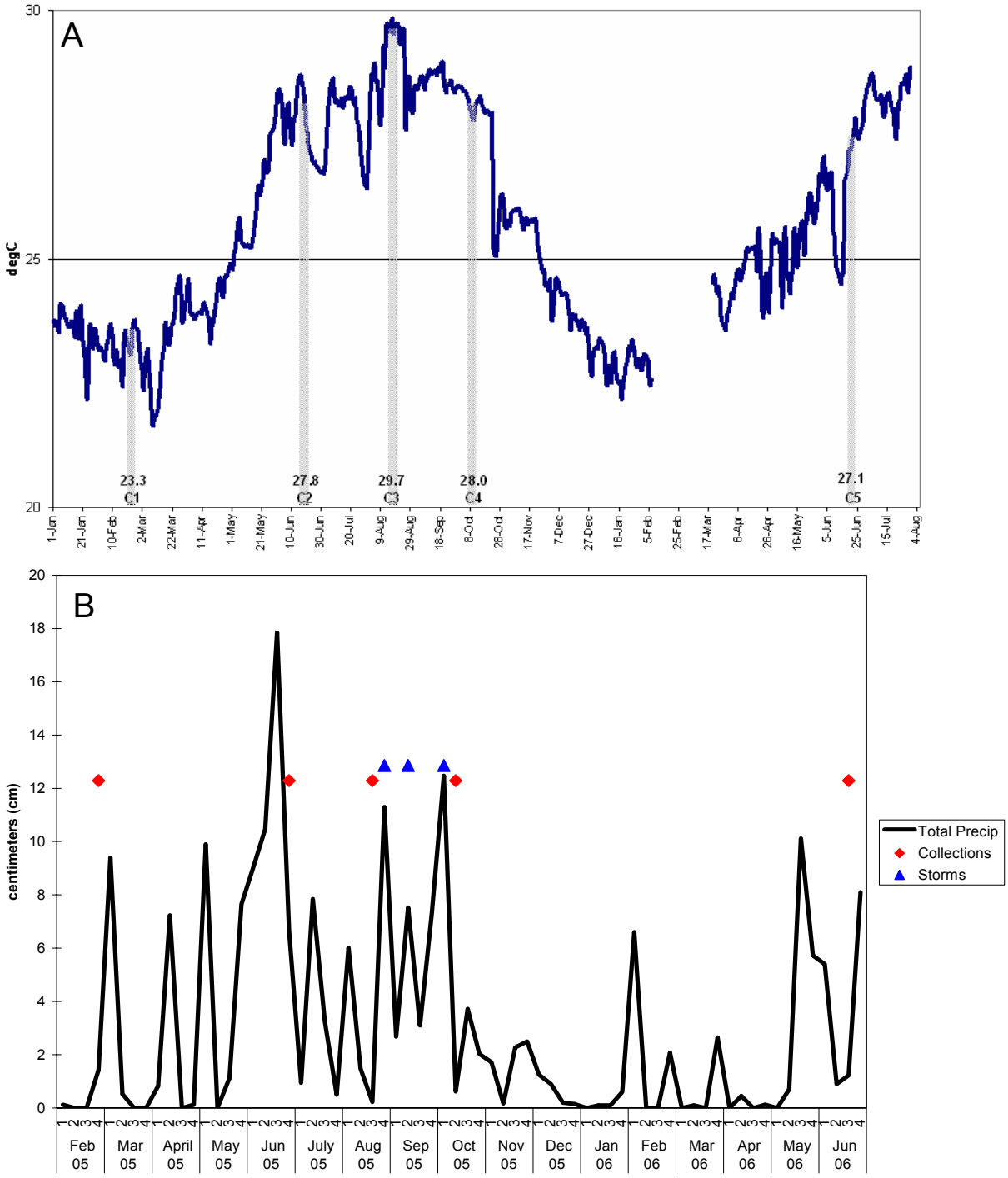


Figure 4.2. **A)** Temperature data from January 2005 through June 2006. Data were collected from 20 meters near site 1 using an Acoustic Doppler Current Profiler (ADCP). Mean temperature for each collection are indicated in degrees Celsius.
B) Precipitation and storm data acquired from the NOAA National Data Center, recorded hourly by the Miami, FL Dade County station (COOPID 85663). Data equals weekly total. Red diamonds are collection dates. Blue triangles are hurricanes Katrina and Wilma, and tropical storm Ophelia.

Five colonies of the scleractinian coral *Montastraea cavernosa* were marked with a numbered tag, mapped and photographed at each site. A small piece (~1 cm^2) was sampled from the lower margin of a colony and preserved in TRIzol® (Invitrogen Inc.) for subsequent RNA processing. Samples were collected five times beginning in February 2005 (22^{nd} – 24^{th}), when samples were collected from sites one through four. In June 2005 (20^{th} – 22^{nd}) we established site five and colonies from all sites were sampled. The remaining collections occurred in August (16^{th} – 18^{th}) and October (10^{th} – 13^{th}) 2005, and June (19^{th} – 22^{nd}) 2006. Due to the loss of several coral colonies to mechanical damage or bleaching, only three colonies per site were analyzed for gene expression.

RNA Isolation and Processing

Total RNA was isolated from a 2 ml aliquot of each preserved coral fragment following the manufacturer's protocol for TRIzol® (Chomezynski and Sacchi, 1987). RNA was purified using a silica-gel–membrane column (Qiagen, Inc.) and concentrations were estimated by ultraviolet absorbance at 260 nm. Integrity of the ribosomal subunits was confirmed by electrophoresis on a 1% formaldehyde agarose gel. Replicate aliquots of at least 5 ug of purified total RNA from each sample collection were reverse transcribed using ImpromII™ reverse transcriptase (Promega, Inc.), an oligo (dT_{16}) primer and a random (9-mer) primer. Before transcription, two spike control mRNAs (*Arabidopsis thaliana* NAC1 and RCP1 0.25 ng each; Stratagene, Inc.), an oligo-dT_{16} primer and random (9-mer) primers were added to purified total RNA. The RNA mix was incubated at 80°C for 10 minutes and chilled on ice. Transcription conditions consisted of incubation at room temperature for 5 min followed by 42°C for 2 hrs, 70°C for 15 min,

and a final incubation at 95°C for 5 min. The reaction was stopped and RNA was hydrolyzed by adding 0.5 M EDTA (pH 8), 1 N NaOH and incubated at 65°C for 15 minutes. The reaction was neutralized by adding 1 M Tris-HCl (pH 7.5). The resulting cDNA was purified with isopropyl alcohol precipitation (IPA) and quantified with a Nano-drop® spectrophotometer. During reverse transcription, aminoallyl-modified dUTPs (5-[3-aminoallyl]-2'-deoxyuridine-5'-triphosphate; Invitrogen, Inc.) were incorporated into the transcribed cDNA and subsequently reacted with an excess of an amine-reactive fluorescent dye (Alexa Fluor 546; ARES Amino-allyl Labeling Kit, Invitrogen, Inc.). The resulting fluorescently labeled cDNA was IPA purified and quantified. Samples with a frequency of incorporation (FOI) of between 20 and 40 were subsequently used as targets to assess changes in gene expression using an Anthozoan oligonucleotide microarray.

Hybridization and Imaging

Fluorescently labeled cDNA from each sample was hybridized to two replicate microarrays. Approximately 1 ug of fluorescently labeled cDNA was added to a hybridization solution consisting of 1 X SSPE buffer, 10% Tween-20, 0.5M EDTA, denatured salmon sperm, and 1% SDS. Arrays were hybridized at 50°C for 12 to 16 hours and washed according to the manufacturer's protocol (Combimatrix, Inc.). A Perkin-Elmer scanner was used to detect the fluorescence of each spot and software provided by CombiMatrix (Microarray Imager) was used to measure signal intensities. After signal detection, arrays were stripped to remove the fluorescently labeled cDNA using a kit provided by the manufacturer (Combimatrix) and re-scanned to assess

stripping efficiency. New samples were hybridized to the stripped arrays and the process was repeated. Each array was used a minimum of four times and cDNA from the same sample was not used more than once on a single array.

Anthozoan Microarray Production

Microarrays were spotted by Combimatrix, Inc. and consisted of 2240 features. One to five oligonucleotide sequences (35 – 40 bases) from different regions of 148 genes were replicated three times on the array to generate 742 probes. In addition, complimentary sequences for the two *Arabidopsis* spike control mRNAs and a negative phage control were included on the array. More than 50 genes represented on the array were isolated from *Montastraea faveolata* and *Acropora cervicornis* exposed to natural or anthropogenic stressors in our lab (Morgan et al., 2001; Morgan and Snell, 2002; Edge et al., 2005). The remaining genes were identified using a bioinformatics approach and literature search. The resulting array consisted of Anthozoan genes involved in a variety of cellular functions, ranging from metabolism and development, to the regulation of apoptosis and the stress response. Refer to the appendix for a comprehensive overview of the array (Appendix, Table A.1) and for a list of the number of genes from each species on the array (Appendix, Table A.2).

Functional Grouping of Genes

Array genes were grouped into 27 categories based on their primary cellular function according to published research and the Gene Ontology database (http://www.geneontology.org). Functional categories were grouped further to provide an

overview of the coral response. The groups include normal cellular function (NCF), multifunctional response (MF), stress response (SR) and symbiont specific response (ZOOX) (Table 4.1). Normal cellular functioning genes are involved in transcription and translation, cellular respiration, metabolism, and signal transduction. Multifunctional response genes span a range between normal cellular functioning and stress response. These include molecular chaperones, such as heat shock elements, and genes involved in the regulation of apoptosis, proteolysis and metal ion regulation. Genes in the stress response category include those involved in DNA repair, wound healing, oxidative stress, and xenobiotics exposure. Some genes in this grouping are uncharacterized with respect to cellular function but were isolated specifically in response to direct exposure to stress (Morgan et al., 2001; Morgan and Snell, 2002; Edge et al., 2005). Finally, the symbiont specific genes are involved in flagellar motility, carbon dioxide fixation, metabolism, growth and development, proteolysis and response to light.

Table 4.1. Number of genes in each functional category and the grouping of the categories.

Function	# of Genes	
regulation of trascription	4	Normal Cellular Function (NCF)
translation	4	
nucleic acid modification	5	
protein modification	7	
protein transport	6	
signal transduction	8	
cellular respiration	6	
metabolism	11	
bioluminescence	1	
cell migration	3	
growth & development	19	
lipid transport / reproduction	1	
regulation of apoptosis	6	Multifunctional (MF)
molecular chaperones	12	
proteolysis	7	
metal ion regulation	9	
DNA repair	1	Stress Response (SR)
oxidative stress	12	
response to xenobiotic	10	
wound healing	3	
unknown (dark expsoure)	1	
zoox - cell motility	1	Symbiont Specific (ZOOX)
zoox - CO2 fixation	2	
zoox - growth & development	1	
zoox - metabolism	1	
zoox - proteolysis	1	
zoox - response to light	6	

Data Analysis

-Tranformation and Normaliztion

The image analysis software, Microarray Imager (Combimatrix, Inc.), automatically subtracts the background intensity from each spot and averages replicate oligonucleotide features to produce a fluorescence intensity measurement for each probe. Before analyzing the data, arrays were visually inspected for hybridization irregularities using

the image analysis software. Inconsistent spots were selected and the anomalous data was discarded by the software before extracting raw numbers. All remaining analyses were performed using JMP Genomics (SAS Institute, Inc.). Data were log base 2 transformed and normalized using a global loess smoothing model. Loess normalization is a curve-fitting technique based on local regression to a between array average (Cleveland and Devlin, 1988; Edwards, 2003). The distribution of the resulting normalized data was assessed. Principle component plots and correlation scatterplots were generated to check the multivariate structure of the normalized data.

-Data Quality Assessment

Numerous factors can affect the quality of data generated in a microarray experiment. These factors may include different reverse transcription and labeling efficiencies, gene-specific variation, minor slide defects, and different hybridization conditions. Therefore, it is important to assess the quality of the data before and after normalization. I used kernel density estimated curves, or parallel plots, to show the univariate distributions of all 72 arrays and box plots to allow comparison of all variables between arrays, such as differences in variance. After log2-transformation and loess normalization, the distributions are much more consistent and differences in variance between arrays are reduced (Figures 4.3a and 4.3b). Principal component analysis and correlation scatterplots confirm these results and reveal minimal variance between colonies (2.19%) compared to the variance between sites (18.43%) (Appendix, Figure A.1). Principal component plots also reveal patterns in the data based on similarity between arrays in terms of Mahalanobis distance. Correlation scatterplots confirm the homogeneity of

samples by plotting each probe on the array and comparing the distribution of probes between arrays. The bivariate normal density ellipses of these scatterplots are thin, diagonal and incorporate more than 95% of the data points revealing tight correlations and normality of the data.

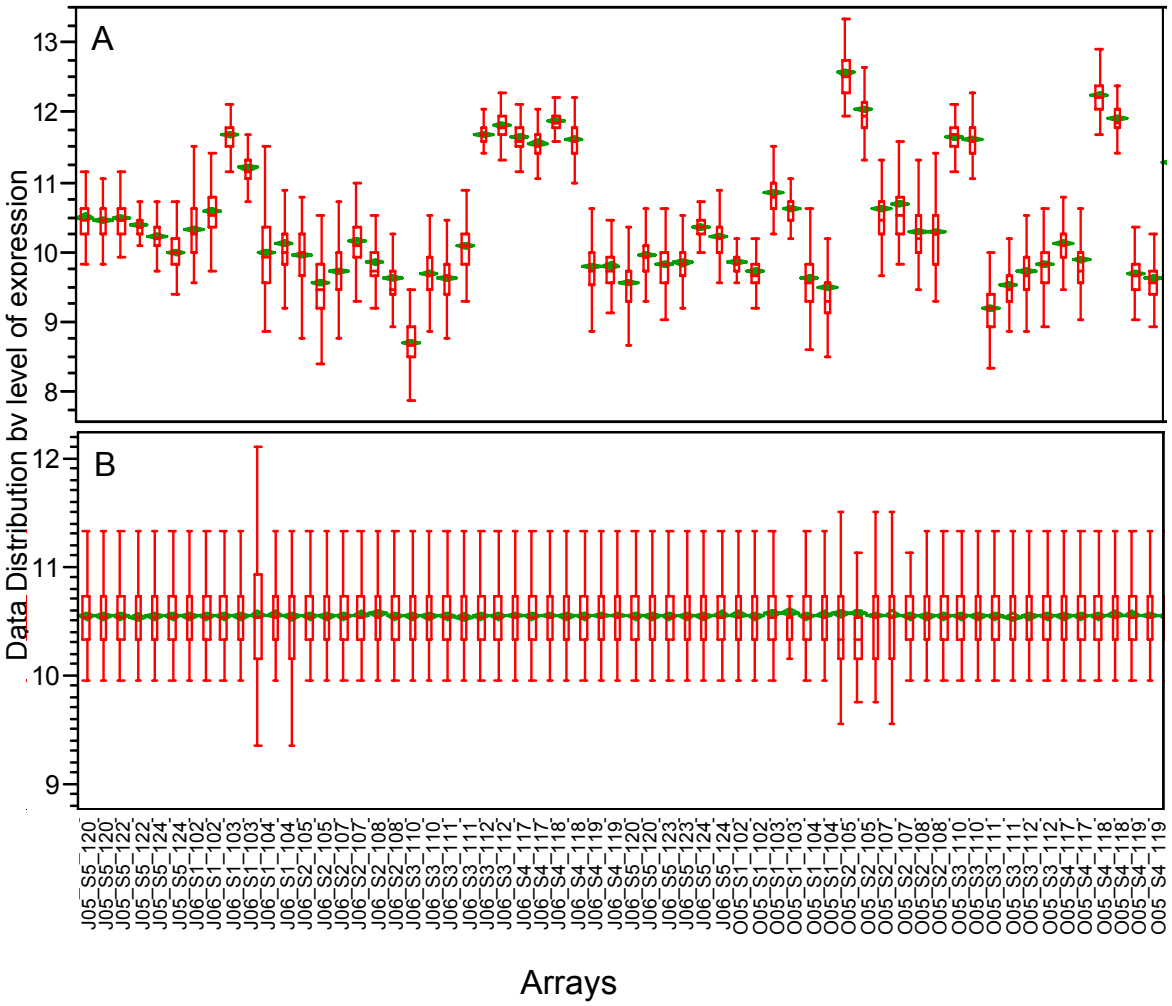


Figure 4.3. Quantile box plot distributions of a subset of the 72 arrays. Each point represents one array, green dashes are the mean of data within an array, whiskers show the range of the data and the box indicates the 25th, 50th and 75th percentiles.
A) log2 transformed data, **B)** log2 transformed and loess normalized data.

-*Statistical Analysis*

Replicate probes for each gene were averaged and a multivariate, repeated measures analysis of variance (corresponding to gene by gene comparisons) was used to quantify significant differences in gene expression. The model was set up with fixed effects and least squares effects as date (n = 5), site (n = 5) and an interaction between date and site (date x site, n = 24). Colony was assigned as a random effect to account for the minimal variance associated with this variable. The cut-off value for significance was set at $-\log10(p) > 3$, which corresponds to a significance of $p < 0.001$. As an adjustment for multiplicity of testing in the investigation of these 148 genes, a method was used to control the false discovery rate at $Q = 0.05$ (Benjamini and Hochberg, 1995). This method leads to a smaller list of genes which may be declared significant. Finally, residual diagnostics indicated linearity and normality of the data validating the fit of the model.

Least squares profiles of the significant genes generated by ANOVA were standardized to a mean of zero. Deviations from the standardized least squares mean (StdLSmean = 0) for significant genes within a functional category were averaged, graphed and analyzed by ANOVA to measure the magnitude of expression between functional responses among coral samples. This was also done between groups of genes. If a significant difference was detected ($p \leq 0.05$), Tukey-Kramer HSD (honestly significant difference) was used to determine which categories or groups were different. Finally, the median of the deviations from the StdLsmean for the four gene groups (NCF, MF, SR, and ZOOX) was calculated per sample, graphed and a correlation analysis was preformed. These

comparisons reveal the level of expression (elevated, no difference, or decreased) between samples based on the functional responses of significant genes and provide an overview of responses by gene groups.

Results

ANOVA Results between samples

The ANOVA analyzing date and site effects identified 135 genes whose expression significantly changed in at least one comparison ($-\log10(p) > 3$, $p < 0.001$). Hierarchical clustering of the standardized least squares means by Ward's method revealed ten clusters of differentially expressed genes and six clusters between samples (Appendix, Figure A.4.2). Ward's method computes cluster proximity by the squared Euclidean distance between the gene cluster mean profiles (Ward, 1963). Expression patterns for June (2005) and October, sites 1 and 2 (J51, J52, O1, O2), are comparable and make up the first cluster. June (2005) and October site 3 (J53, O3) also show similar expression profiles and comprise the second cluster. The third cluster consists of August and June (2006) site 2 (A2, J62), and February site 4 (F4). The fourth cluster includes August site 4 (A4), February site 3 (F3), and site 4 from June (2005), June (2006) and October (J54, J64, O4). Cluster five includes August site 3 (A3), February site 1 (F1), and October site 5 (O5). The remaining samples make up the final cluster but exhibit slightly higher variability in expression between samples than seen in other clusters (A5, J65, A1, J61, J55, and J63). February site 2 (F2) did not cluster with any other sample and exhibits a unique expression pattern.

Comparison of group response (NCF, MF, SR and ZOOX) by cluster

--Clusters 1 and 2

Although the hierarchical clustering map groups samples J51, J52, O1 and O2 and places J53 and O3 in a separate cluster, they reveal similar expression patterns. Both clusters show a significant difference between groups (cluster 1, $p < 0.0001$; cluster 2, $p = 0.0473$; Figure 4.4). Genes involved in normal cellular functioning (NCF) are significantly decreased in cluster 2, but do not deviate from the mean level of expression in cluster 1. Multifunctional genes (MF) are not significantly different from normal cellular functioning genes in either cluster. Genes in the stress response group (SR) appear elevated in both clusters but are only significantly different from the normal cellular functioning genes (NCF) in cluster 2. Symbiont-specific genes (ZOOX) are significantly elevated compared to the normal cellular functioning (NCF) and multifunctional (MF) genes in cluster 1, but are only different from the normal cellular functioning genes in cluster 2. In general, these patterns are reflected for individual samples in cluster 1 (J51, J52, O1, O2), but samples in cluster 2 (J53, O3) show no significant difference between groups (Appendix, Figures A.3a).

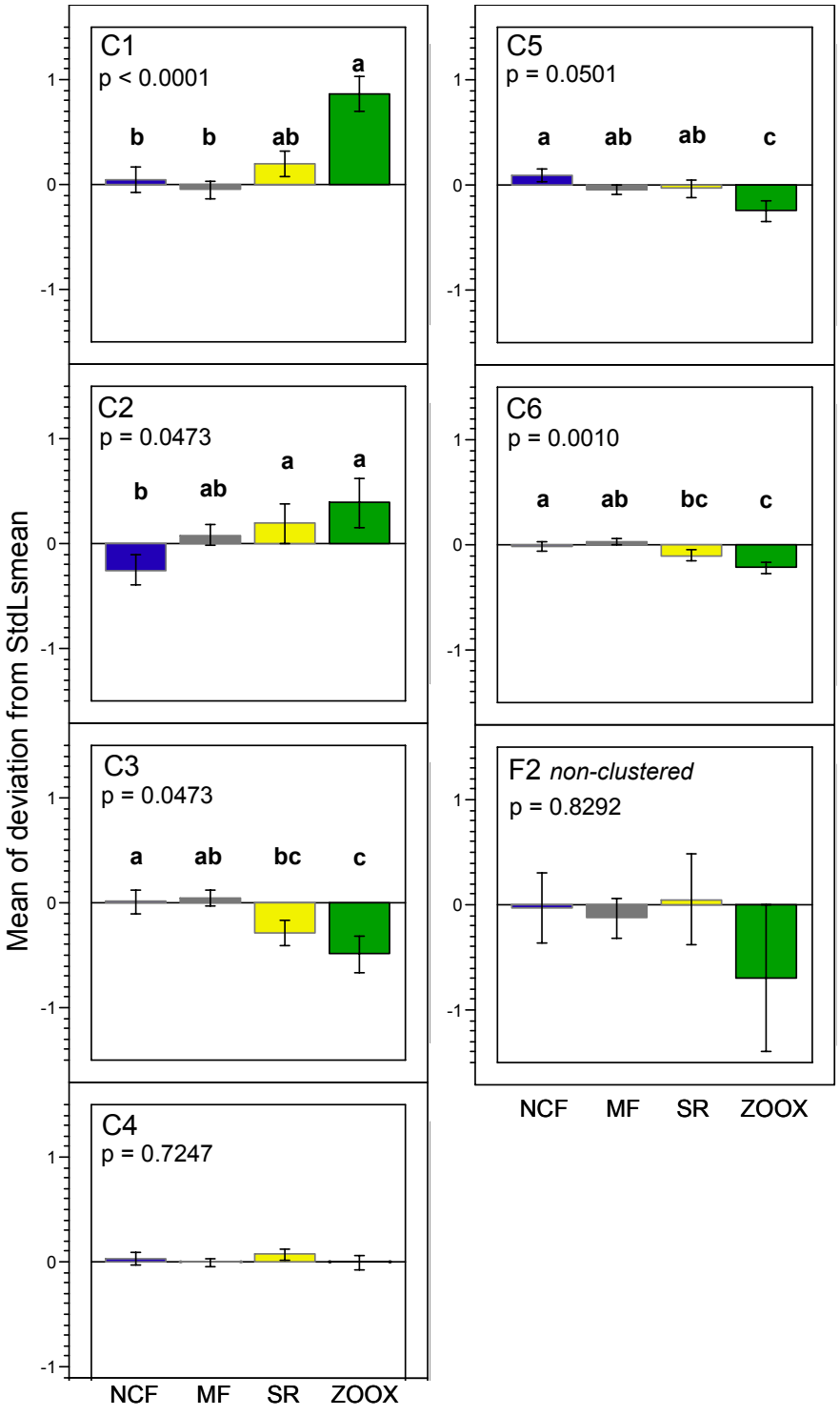


Figure 4.4. Deviation of significant genes from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning (NCF), multifunctional (MF), stress response (SR), and symbiont specific (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different cluster.

--*Cluster 3*

This cluster contains the samples F4, A2, and J62. Groups in this cluster are significantly different ($p = 0.0473$, Figure 4.4). The symbiont specific group (ZOOX) is significantly decreased compared to the normal cellular functioning (NCF) and multifunctional groups (MF), but is not statistically different than the stress response group (SR). The stress response group (SR) is significantly decreased compared to the normal cellular functioning group (NCF) (Appendix, Figure A.3b). When gene groups are compared between individual samples, F4 and J62 show significant differences between groups ($p = 0.0500$ and $p = 0.0492$ respectively). However, A2 shows no significant differences ($p = 0.2345$; Appendix, Figure A.3b).

--*Cluster 4*

Samples F3, A4, J54, J64, and O4 are in this cluster and none of the groups (NCF, MF, SR, and ZOOX) are significantly different by cluster or by sample ($p = 0.7247$; Figure 4.4 and Appendix, Figure A.3c).

--*Cluster 5*

F1, A3 and O5 are included in this cluster and differences between the groups are marginally significant by ANOVA ($p = 0.0501$), but the Tukey-Kramer test indicates that the symbiont specific group (ZOOX) is statistically decreased compared to the other groups (Figure 4.4). When group response is compared by sample, F1 and O5 reveal no significant differences ($p = 0.1857$ and $p = 0.3195$ respectively). However, the symbiont

specific group in sample A3 is statistically lower compared to other groups ($p = 0.0130$; Appendix, Figure A.3d).

--*Cluster 6*

This cluster consists of samples A1, A5, J55, J61, J63, and J65. Groups are significantly different within this cluster ($p = 0.001$). The symbiont specific (ZOOX) and stress response (SR) groups are significantly decreased compared to the normal cellular functioning group (NCF) (Figure 4.4). However, when individual samples are analyzed, only J65 and A5 show a significant difference between groups ($p = 0.0501$ and $p = 0.0004$ respectively) and exhibit the same response as the overall cluster (Appendix, Figure A.3e).

--*Non-clustered gene*

February, site 2 (F2) does not cluster with any other sample reveals no significant differences between groups ($p = 0.8292$; Figure 4.4).

ANOVA results by date and site

The multivariate ANOVA between dates identified 98 genes whose expression significantly changed in at least one comparison while the ANOVA between sites identified 114 significant genes ($p < 0.0001$). For the date comparison, hierarchical clustering of the least squares means revealed nine clusters between genes and two clusters between dates. Among dates, June (2005) and October form one cluster and a second cluster consists of February, August and June (2006) (Appendix, Figure A.4).

Hierarchical clustering of the site data produced eight clusters of genes and three clusters between sites. Sites four and five (S4, S5) showed the tightest clustering, followed by sites one and three (S1, S3). Site 2 (S2) stands apart and does not cluster strongly with any other site (Appendix, Figure A.5).

The ANOVA results for date and site effects are consistent with results from the analysis of date and site (i.e. sample effect) based on hierarchical clustering of genes and effects (Appendix, Figures A.2, A.4, and A.5). For example, June (2005) and October cluster by date, sites 1 and 3 clusters by site and J51, J53, O1 and O3 cluster by sample. Similar results are produced for August, June (2006), site 4 and site 5. Finally, February clusters distantly with other dates, site 2 does not cluster with other sites, and F2 does not cluster with any other sample.

Observed Patterns in Grouped Data

--*Stress Response and Symbiont-Specific groups*

Stress response (SR) and symbiont specific (ZOOX) groups reveal significantly different responses across dates and sites ($p = 0.0205$ and $p < 0.0001$). These groups are elevated in June (2005) and October, but are decreased in February, August and June (2006). However, at site 2 in February, expression of the symbiont-specific group (ZOOX) is significantly decreased. Sites 1, 2 and 3 reveal elevated expression, but this pattern only occurs during the months of October and June (2005) (Figure 4.5).

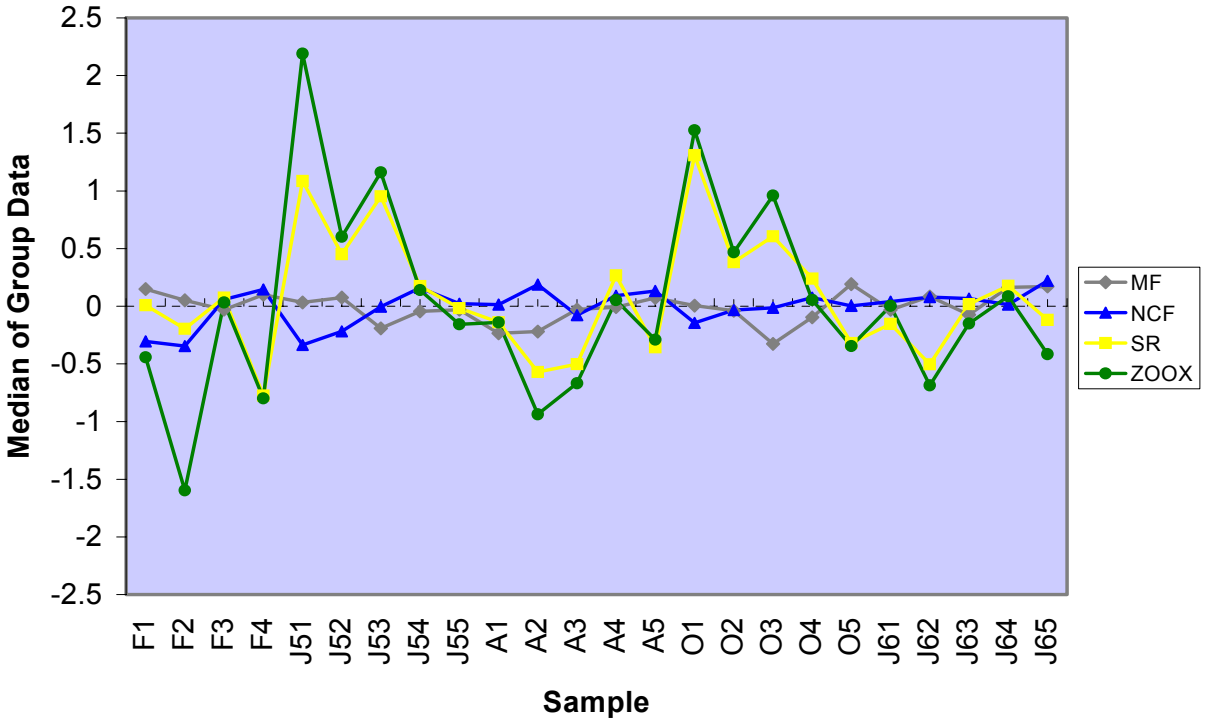


Figure 4.5. Overview of response by group; normal cellular functioning (NCF), multifunctional (MF), stress response (SR), and symbiont specific (ZOOX) genes. The median values of deviations from the StdLsmean for genes within a group were graphed by sample. Statistical differences between samples were compared by ANOVA. NCF ($p = 0.9970$); MF ($p = 0.8740$); SR ($p = 0.0205$*); ZOOX ($p < 0.0001$*). Correlation analysis compared group responses SR/ZOOX = 0.90; NCF/MF = -0.16; all groups = -0.37. *An alternate version of this graph by cluster is located in the appendix, Figure A.6.*

--*Normal Cellular Function and Multifunctional groups*

Compared to the stress response (SR) and symbiont-specific (ZOOX) groups, the multifunctional (MF) and normal cellular functioning (NCF) groups reflect an oscillatory pattern of expression but do not deviate significantly from the mean. These groups are not statistically different between dates and sites ($p = 0.9970$ and $p = 0.8740$; Figure 4.5).

-*Correlation Analysis*

Genes in the stress response (SR) and symbiont specific (ZOOX) groups correlate strongly (0.9), while the multifunctional group (MF) and normal cellular functioning

(NCF) group show a slight negative (-0.16). Across all groups the correlation value is -0.37 (Figure 4.5; see Appendix, Figure A.6 for an alternate version of this graph).

Specific Gene Comparisons

Significantly different genes identified by ANOVA were compared on an individual basis between samples. The degree of deviation from the standardized least squares mean (StdLsmean = 0) was used to determine the magnitude of expression, and whether gene expression was elevated or decreased in a sample. The majority of genes (72.5%) fall within +/- 1.0 arbitrary units of the mean, and the greatest deviations occur at a magnitude of |4| (Appendix, Figure A.7 – histogram). Only genes exhibiting deviations of greater than or less than 1.0 arbitrary unit from the StLsmean were considered in the gene by gene analysis. Results of this analysis are summarized for a subset of the significant genes in table 4.2. In addition, the differences between the deviations from the StdLsmean per gene were plotted against the -log10(p-value) for the samples being compared. This type of graph is known as a volcano plot and shows the degree of differential expression per gene between samples as well as the level of significance for a given gene (Appendix, Figure A.8). Due to the large number of plots (276) created by these comparisons, a single, representative graph comparing two of the samples is included.

Table 4.2. Deviations from the standard least squares mean (StdLsmean = 0) for a subset of significant genes produced by multivariate ANOVA between samples. The majority of genes (72.5%) fall within |1.0| units of the StdLsmean. Genes that deviate by ~ +/- 1.0 units are highlighted. Red indicates a positive deviation from the StdLsmean (elevated) and green a negative deviation (decreased). § Indicates no accession number since the gene sequence has not yet been submitted to Genbank.

Function	Gene Name	Accession	F1	F2	F3	F4	J51	J52	J53	J54	J55	A1	A2	A3	A4	A5	J61	J62	J63	J64	J65	O1	O2	O3	O4	O5
cellular respiration	cox I	AF013738	0.63	0.18	0.03	1.21	-2.41	-1.98	1.71	0.27	0.28	1.80	-0.09	0.32	-0.17	0.66	-0.34	-0.30	0.34	-0.07	0.61	-1.02	-1.38	-0.81	0.08	0.47
	NADH-ubiquinone dehydrogenase	P34943	0.48	1.37	-0.33	1.93	-1.09	0.39	-1.15	-0.17	-0.25	-0.16	0.83	1.21	-0.76	0.55	-0.01	0.98	0.16	-0.62	0.34	-2.83	0.39	-1.21	-0.25	0.22
	NADH-ubiquinone dehydrogenase	DQ351254	0.35	-0.57	1.04	0.65	-2.38	-1.60	-1.47	0.37	0.22	0.43	0.49	0.04	0.63	0.79	0.00	1.33	0.98	-0.71	0.57	-0.80	-1.20	-1.18	0.93	1.10
	NADH-ubiquinone dehydrogenase	NP_998304	-0.11	-1.02	0.98	-0.02	-1.32	-1.23	0.67	0.45	-0.16	-0.63	-2.01	0.11	0.36	1.17	-0.02	-0.17	1.21	1.31	0.95	-1.74	-1.22	1.38	0.40	0.65
metabolism	acyl-CoA thioesterase	DQ309537	0.23	4.25	-0.69	0.61	0.33	0.29	-0.81	-0.08	-0.61	-0.73	0.12	0.00	-0.59	-0.44	-0.42	-0.18	-0.29	-0.29	0.33	-0.42	0.26	-0.83	-0.42	0.39
	acyl-CoA thioesterase	DQ309539	0.95	2.94	-0.68	-0.94	0.33	-0.36	0.39	0.73	-0.10	-0.71	-0.23	0.54	0.55	-1.09	-0.38	-1.47	-1.56	0.64	-0.26	0.81	-0.65	-0.73	-0.12	1.40
bioluminescence	green fluorescent protein	AY181557	-0.54	-1.38	0.91	-0.90	-1.59	-1.56	-0.19	1.43	0.21	-0.12	-1.51	-0.55	1.15	0.79	0.08	0.65	0.50	1.10	0.69	-0.27	-1.48	0.74	1.50	0.34
regulation of apoptosis	BCL-2 antagonist/killer	CN774282	0.21	1.52	-0.04	0.91	-1.32	-0.36	-2.20	-0.41	-0.21	0.46	1.90	0.36	0.11	0.61	-0.11	1.34	0.02	-0.36	0.87	-1.54	0.00	-1.87	0.03	0.05
	BCL-2 apoptosis regulator	CV181079	0.67	1.19	-0.32	2.15	-1.96	-0.56	-1.06	-0.44	-0.04	0.57	1.41	0.96	-0.17	0.22	-0.14	1.09	-0.19	-0.70	0.22	-1.99	-0.43	-1.00	-0.27	0.77
	cystatin	BC020532	-0.14	0.50	0.52	-0.62	-2.02	-1.83	0.67	0.33	0.43	0.96	0.51	0.44	0.76	0.30	0.38	0.00	0.27	0.45	0.65	-2.89	-1.27	0.88	0.46	0.26
	NALP; zinc finger protein; Cryopyrin	AAO18165.1	-0.20	-1.63	0.48	-1.05	-0.84	-1.26	-2.72	1.02	0.24	0.32	-0.15	-0.49	0.69	1.36	0.82	-0.28	0.21	0.14	0.14	1.87	-0.33	0.21	0.79	0.65
molecular chaperone	Hsp 70	Ac_28§	-0.34	0.02	-0.39	1.40	-2.02	-1.29	0.46	1.55	0.02	-0.48	-1.49	0.36	0.36	0.88	-0.27	-0.34	0.36	1.39	-0.52	-1.68	-0.78	0.64	1.42	0.73
	glucose regulated protein	MGID_114	-0.72	-2.35	0.93	-1.20	0.68	-0.06	1.34	1.10	0.18	-0.04	-1.11	-0.79	0.61	-0.49	-0.15	-1.24	-0.61	0.72	-0.37	1.70	-0.46	1.22	1.23	-0.10
proteolysis	polyubiquitin	BAA09860.1	-0.39	-2.47	0.40	-0.90	1.54	0.52	0.34	0.79	0.51	-0.38	-1.04	-0.57	0.72	-0.52	-0.48	-1.71	0.05	0.70	-1.06	1.87	0.23	0.83	0.88	0.15
	RING-H2 finger protein	AP000616	0.29	0.53	0.70	0.64	-2.33	-2.02	-0.01	0.61	0.38	0.21	0.49	0.49	0.51	0.51	0.27	0.58	0.50	0.44	0.23	-2.32	-1.92	0.07	0.65	0.50
	ubiquitin	DN252355	-0.14	2.51	0.13	-0.08	-1.35	-1.39	1.41	1.30	0.18	-0.51	0.57	0.22	-0.04	-0.56	-1.36	-0.81	-1.08	1.22	0.32	0.42	-1.40	-0.55	0.37	0.63
metal ion regulation	ferritin	AJ306614	-0.41	-1.67	-0.14	-0.52	2.45	1.21	1.29	-0.06	-0.23	-0.33	-1.56	-0.53	-0.30	-0.52	0.15	-1.16	-0.08	0.27	-0.84	1.70	1.16	0.81	-0.12	-0.56
	metallothionein	CV564390	0.37	0.28	-0.30	1.06	-3.17	-1.47	1.20	0.86	0.19	-0.27	-0.02	-0.11	-0.07	0.06	0.27	0.44	-0.03	0.03	1.07	-1.12	-1.57	0.83	0.47	0.99
	metallothionein	DR681654	1.12	1.52	0.31	0.72	-1.63	-0.84	-1.86	0.00	0.22	0.30	0.61	0.90	0.34	-0.05	0.27	1.03	0.31	-0.29	0.09	-2.64	-0.62	-0.79	-0.16	1.12
	Ceruloplasmin homolog	DN167139	0.93	2.30	-0.62	2.83	-0.26	0.95	-0.68	0.06	-0.52	-0.73	-0.10	0.42	-0.38	-0.85	-1.08	-0.34	-0.32	0.24	-0.28	-1.39	0.79	-1.02	0.13	-0.08
	cytochrome b	AB117374	0.66	3.91	-0.61	1.25	-0.67	-0.05	-0.22	0.07	-0.17	-0.11	0.29	0.22	-0.82	-0.63	-0.72	0.44	-0.34	-0.45	0.21	-0.98	-0.52	-0.68	-0.69	0.60
	DHSB protein	AJ251055	0.52	-1.35	-0.03	0.33	-0.54	-0.37	-0.52	0.55	0.26	-0.81	-3.14	0.48	-0.23	0.57	0.22	1.32	0.78	1.14	1.82	-0.65	-0.82	-0.35	0.26	0.55
DNA repair	N-methylpurine DNA glycosylase	AF499437	-0.09	-1.65	0.52	-0.25	1.09	0.15	1.24	0.91	-0.02	-0.86	-2.32	-0.68	0.54	-0.36	-0.41	-1.66	0.06	1.25	-0.64	1.50	0.02	1.26	0.73	-0.34
oxidative stress	glutathione peroxidase	AY836663	0.02	-1.18	-0.33	-1.38	0.08	-0.49	2.67	0.45	0.58	-0.16	-1.56	-0.80	-0.33	-0.72	0.64	-0.80	1.47	-0.30	-0.31	1.38	-0.44	1.55	0.13	-0.18
	glutaredoxin	P55143	0.12	-1.76	-0.21	0.06	2.72	1.07	0.42	-0.88	0.09	-0.24	1.13	-0.74	0.20	0.11	-0.74	-1.70	-0.97	0.23	-0.07	0.81	1.63	-0.62	-0.11	-0.54
	peroxiredoxin	MGID_12	-0.70	1.41	-1.26	-0.54	1.17	1.21	-0.31	1.48	-0.29	-0.74	-0.74	-0.90	-0.76	-0.93	-0.71	-0.63	-0.51	-0.52	0.94	2.61	0.97	-0.17	-0.03	-0.07
	copper/zinc SOD	DQ309550	0.80	2.29	-1.29	-0.71	1.20	1.26	-1.14	-1.02	0.09	-0.08	0.90	-0.23	-0.93	-0.69	-0.91	0.31	-0.03	-0.65	1.16	0.95	0.86	-1.10	-1.32	0.26
	copper/zinc SOD	AY164664	0.60	2.88	-0.03	0.15	1.09	1.35	-1.35	-0.36	-1.19	0.15	0.88	0.42	-0.75	-0.62	-0.10	-0.03	0.01	-0.46	0.78	-0.33	0.53	-1.71	-1.03	-0.91
	copper/zinc SOD	U27840	0.17	-0.75	-1.19	-1.03	2.54	1.09	-0.24	-0.35	-0.17	-0.13	0.06	-1.10	-1.15	-0.62	-0.44	-0.58	0.22	-0.81	0.51	1.81	1.18	1.66	-0.36	-0.31
	selenium binding protein	NP_543168	-1.05	-1.87	0.67	-1.02	-1.00	-1.10	1.60	1.49	0.25	0.48	-0.41	-0.78	1.08	-0.34	0.62	-0.96	-0.31	0.80	-0.63	0.83	-0.85	1.53	1.15	-0.17
response to xenobiotic	copper exposure	C-5G28§	1.01	3.64	-0.42	0.42	-0.52	0.02	-1.88	-0.03	-0.17	-0.63	0.09	0.52	0.19	-0.09	-0.56	-0.18	-0.36	0.16	0.13	-0.41	-0.14	-1.50	-0.23	0.94
	copper exposure	BI534458	1.04	3.14	-0.29	1.63	-0.91	0.13	-1.27	0.23	0.15	0.19	0.03	0.44	0.36	-1.16	-1.10	0.27	-0.51	-0.08	-0.38	-0.10	-0.33	-1.77	-0.04	0.35
	dibrome	BI534456	1.08	2.22	-0.09	0.79	-1.80	-0.82	-1.81	0.15	-0.08	0.37	0.98	0.76	-0.10	-1.01	0.67	1.50	-0.25	-0.68	-0.24	-1.46	-0.14	-0.74	0.06	0.62
	permethrin exposure	P+3G22	0.93	3.66	-0.46	0.07	-0.89	-0.64	0.12	0.06	-0.11	-0.63	0.53	0.40	-0.08	-0.08	-0.63	-0.61	-0.58	0.90	0.55	-1.18	-1.12	-0.78	-0.35	0.94
	mercury	BI534459	0.18	1.62	0.05	1.24	-2.37	-1.42	-1.40	-0.41	0.73	0.27	1.09	0.67	0.24	0.69	-0.70	0.99	0.10	0.01	0.69	-0.15	-0.94	-1.79	0.24	0.36
	glutathione-s-transferase	AAT09082	-0.51	-1.58	2.42	0.53	-0.69	-0.39	-1.28	-0.55	-0.13	1.02	-0.55	1.77	0.91	0.92	0.43	0.71	0.54	0.65	-1.45	-0.85	-0.33	-0.53	-0.19	-0.88
	glutathione-s-transferase	BC005964	0.59	-1.87	0.93	-2.08	-0.89	-1.12	1.37	0.61	0.25	0.26	1.03	-0.65	0.94	0.40	-0.50	-1.64	-0.21	-0.02	0.54	0.31	-0.84	1.43	0.89	0.27
wound healing	cysteine rich FGF receptor	U28811	0.81	3.07	-0.21	-0.25	1.23	0.27	-0.18	-0.07	-0.53	-0.35	0.25	0.91	0.09	-1.08	-1.00	-1.61	-1.38	0.77	-0.33	0.78	-0.13	-0.97	-0.72	0.63
	Ly-6; uPAR; single pass membrane protein	CDD|14821	-0.54	-1.73	-0.09	-0.99	1.45	1.05	1.65	0.57	-0.38	-0.47	-0.84	-0.99	-0.11	0.14	-0.62	-1.34	-0.47	0.04	-0.68	2.06	0.88	1.32	0.64	-0.55
	myosin heavy chain	CAA37311	-0.23	-1.93	0.53	-1.87	1.03	-0.08	1.38	0.57	-0.47	0.03	-1.32	0.39	0.83	-1.46	0.21	-1.09	-0.53	0.29	0.66	1.63	0.17	1.47	0.30	-0.50
cell motility	microadriaticum calmodulin (SMCaM1)	AF007889	-0.49	-1.87	0.17	0.22	2.32	1.11	-0.20	-0.03	-0.51	-0.40	-1.10	-0.60	0.16	-0.15	-0.04	-1.17	-0.37	0.11	-0.35	2.69	1.17	-0.07	-0.06	-0.54
CO2 fixation	RuBisCO	AF299359	-0.19	-2.47	-0.22	-1.30	2.10	0.68	0.99	0.15	0.10	-0.61	-1.80	-0.55	-0.05	-0.24	-0.22	-0.45	0.28	0.52	-0.38	1.64	0.71	1.12	0.25	-0.05
CO2 fixation	RuBisCO	AF298221	-0.72	-1.83	-0.62	-0.91	2.11	0.80	1.51	-0.36	-0.59	-0.29	-1.66	-0.31	-0.69	-0.21	0.63	0.06	1.14	-0.10	0.18	1.46	0.67	1.13	-0.82	-0.57
growth & development	actin	AB086828	0.09	-1.95	-0.06	-0.49	2.31	1.25	1.07	0.08	-0.42	-0.69	-0.91	-0.90	0.06	-0.36	-0.40	-1.27	-0.07	0.75	-1.01	1.95	0.73	0.80	-0.17	-0.38
metabolism	glyceraldehyde-3-phosphate dehydrogenase	AB106689	-0.77	-2.46	0.16	-1.55	1.21	0.47	1.98	0.60	0.18	-0.28	0.25	-1.01	0.23	-0.78	-0.05	-0.35	-0.40	-0.12	-0.65	1.50	0.21	1.32	0.97	-0.65
proteolysis	subtilisin/kexin isozyme	AL133583	-0.43	-2.17	0.13	-2.13	2.28	0.42	1.55	0.42	-0.03	0.02	-0.97	-0.74	0.01	-0.14	0.27	-1.02	0.16	0.06	-0.26	0.59	0.21	1.58	0.17	0.02
response to light	ferredoxin	P10770	-0.26	-2.23	0.43	-1.61	1.95	0.80	0.74	0.94	-0.02	0.02	-1.20	-0.53	0.16	-0.99	0.31	-0.34	0.02	0.25	-1.48	1.77	0.44	0.72	0.43	-0.33
response to light	peridinin chlorophyll-a	L13613	-0.72	-1.21	0.24	-1.62	2.50	1.50	1.01	0.39	-0.43	0.01	-0.41	-0.52	0.08	-1.01	-0.40	-0.61	-0.66	-0.05	-1.01	1.60	1.16	0.56	0.40	-0.81
response to light	peridinin chlorophyll-a binding protein	AY149154	1.14	3.57	-1.24	-0.24	0.76	0.35	-1.09	-0.24	-0.05	-0.61	0.44	0.19	-0.12	-0.75	0.10	0.35	-0.67	-0.54	-0.08	0.66	-0.20	-1.46	-0.71	0.45
response to light	photosystem II protein D1 (psbA)	AJ884906	0.50	2.21	0.38	-0.24	-2.31	-1.44	-1.43	0.80	0.31	0.35	1.38	-1.13	0.30	-0.36	0.08	0.97	0.41	-0.16	-0.23	-0.19	-0.74	-0.96	0.54	0.97
response to light	ultraviolet-B-inducible ribosomal protein	AY065657	0.45	2.76	-0.82	1.52	-0.68	0.41	-1.41	-0.88	-0.16	-0.13	0.60	0.34	-0.85	-0.24	0.15	1.33	-0.24	-1.08	0.39	-1.42	0.57	-0.90	-0.62	0.88

-Normal cellular functioning genes

Most of the normal cellular functioning categories, such as regulation of transcription, translation, protein transport and signal transduction are variable between samples and do not show a consistent pattern. However, genes involved in cellular respiration are consistently decreased in June (2005) and October at sites 1, 2 and 3, whereas both acyl-CoA thioesterase genes, involved in metabolism, are consistently elevated above the StdLsmean in February at site 2 (+4.25 and +2.94; Table 4.2 and Appendix, Figures A.9). Expression of the green fluorescent protein gene is decreased at site 2 on all dates, except June 2006, and is increased at site 4 on all dates except February (Table 4.2 and Appendix, Figures A.9).

-Multifunctional Genes

Expression of apoptotic genes, such as BCL-2, are decreased in June (2005) and October at sites 1 and 3 but elevated in February, August and June (2006) at site 2. Molecular chaperones show considerable variability in expression. Of the twelve genes in this category, four do not differ significantly between samples based on the multivariate ANOVA (hsp60, $hsp90_1$, $hsp90_2$, and a small hsp), six show highly variable expression between samples (Hsc71, Hsp 22, Hsp 27, Hsp 90, Hsp 70) and an Hsp 70 gene (*A. millepora*) and a glucose regulated protein gene (*M. annularis*) are elevated at site 4 in June (2005), June (2006) and October. Two of the proteolytic genes, RING-H2 finger and ubiquitin, are decreased at sites 1 and 2 in June (2005), June (2006) and October, while polyubiquitin is increased at site 1 in June (2005) and October. Ubiquitin is elevated in February at site 2, in June (2005) at sites 3 and 4, and in June (2006) at site 4. In general,

metal ion regulation genes are elevated in February at sites 2 and 4. Cytochrome b is considerably elevated at site 2 in February. A DHSB protein gene is distinctly decreased in August and February at site 2, but shows increased expression at sites 2 through 5 in June, 2006. Ferritin is decreased at site 2 in February, August and June (2006), but shows elevated expression at sites 1 through 3 in June (2005) and October. Both metallothionein genes are decreased in June (2005) and October at sites 1 and 2. Finally, a ceruloplasmin homologue is elevated in February, except at site 3. Results are summarized in Table 4.2 and Appendix, Figure A.2.

-*Stress Response*

The DNA repair gene, N-methylpurine DNA glycosylase, is elevated at site 4 in June (2005) and June (2006) and in samples from sites 1 and 3 in June (2005) and October. Overall, oxidative stress genes are elevated in June (2005) and October at sites 1 and 2. However, two of the three copper/zinc superoxide dismutase genes and peroxiredoxin are increased in February at site 2 and decreased at site 3. Copper/zinc superoxide dismutase genes are also decreased at sites 3 through 5 in June (2005) and October. In the xenobiotics category, genes responsive to copper, dibrom, mercury and permethrin exposures are elevated in coral from February, site 2, while all of the glutathione-s-transferase genes are decreased in this sample. The majority of genes in the xenobiotic category are decreased in June (2005) and October at sites 1 through 3. Expression of genes in the wound healing category are elevated in corals from June (2005) and October, sites 1, 2 and 3. Genes in this category are decreased at site 2 in February and June (2006) and in February at site 4. However, the cysteine rich FGF receptor is increased at site 2 in

February. The myosin heavy chain gene and the Ly6/uPAR gene are elevated at site 4, except for in February, and the cysteine rich FGF receptor gene is markedly elevated in February at site 2, and in June (2005) and October at site 1. Results are summarized in Table 4.2 and Appendix, Figure A.2.

-*Symbiont Response*

This group of genes is further divided into six sub-categories. These include response to light, growth and development, metabolism, proteolysis, carbon dioxide fixation, and cell motility (specifically flagellar motility). The most obvious pattern across the group is the elevation of most of the genes in June (2005) and October at sites 1, 2 and 3, and a reciprocal decrease in expression of the same genes in February at sites 2 and 4, and in August at site 2. Of the eleven genes in this group, the only 3 that do not conform to this pattern are in the response to light sub-category and include a peridinin chlorophyll-a binding protein gene, photosystem II protein D1 (psbA), and the ultraviolet-B-inducible ribosomal protein gene. These three genes show an exact opposite pattern of expression. Results are summarized in Table 4.2 and Appendix, Figure A.2.

Discussion

The coral stress microarray includes genes involved in normal cellular functioning and the stress response, as well as multifunctional genes and symbiont-specific genes. Many of the genes have roles in maintaining normal cellular functions, but are also responsive to multiple stressors (molecular chaperones, apoptosis regulators, initiators of proteolysis), while others have more specific responses to stress (DNA repair, wound

healing, antioxidant enzymes). The expression patterns of general and specific genes can be interpreted to indicate the effect of stress on cellular functions and lead to stressor diagnosis (Gasch et al., 2000; Williams et al., 2003).

Previous versions of the array have demonstrated stressor-specific gene expression patterns in laboratory exposures (Edge et al., 2005), detected responses to heavy metals and pesticides at sites near a point source of pollution (Morgan et al., 2005), and tracked changes in gene expression of a coral population over time in the field (Edge et al., *in press*). The current study demonstrates that valuable information can be obtained by comparing different coral populations in the field to determine if they are exposed to similar stress regimes.

Distinctive Gene Expression Patterns

The most distinctive pattern resulting from gene expression analysis of corals from these South Florida sites is a spike in expression of stress genes and symbiont-specific genes during the months of June and October 2005 at sites 1 and 3. Site 2 also shows elevated expression of stress and symbiont genes during these months, but to a lesser degree. This pattern does not appear to correlate with thermal stress because the response does not occur at all sites and is independent of depth. Site 1, the apparently most stressed site, is at a depth of 20 meters, while sites 2 and 3 are at 10 meters. In addition, June (2005) and October are not the warmest months of the collection period. August had the warmest temperatures, averaging near 29 °C, while June (2005), October and June (2006) range between 27 °C and 28 °C. Previous studies using protein biomarkers have not reported

the expression of genes for heat shock elements, an indicator of thermal stress, at temperatures below 33 °C (Black et al., 1995; Sharp et al., 1997; Downs et al., 2000b).

The lack of consistent expression of genes related to thermal stress is another indication that elevated temperature may not be responsible for the observed responses. Of the twelve molecular chaperones on the array, four are not significantly expressed across any of the site/date combinations (Hsp 60, *A. viridis*; Hsp 90, *D. klunzingeri*; Hsp 90, *M. annularis*; and small Hsp, *M. annularis*) and six show highly variable expression between samples (Hsc71, Hsp 22, Hsp 27, Hsp 90, Hsp 70). However, an Hsp 70 gene (*A. millepora*) and a glucose regulated protein gene (*M. annularis*) are elevated at site four in June (2005), June (2006) and October. It is unlikely that the variability in molecular chaperone expression is due to the multi-species origin of the genes. Research has demonstrated low genetic diversity among Anthozoans (van Oppen et al., 1999; Shearer et al., 2002) and the oligonucleotides on the array were selected because they are from highly conserved gene regions so that cross-hybridization among species would be maximized. In addition, conserved genes or proteins have been used in previous cross-species studies successfully (Sharp et al., 1997; Morgan et al., 2005; Lejeusne et al., 2006; Travers et al., 2007). The variability in expression of molecular chaperones is not unusual and reflects the difficulty of interpreting data from field collected specimens. Several studies have revealed or commented on the breadth of the response of heat shock element genes to different stressors (Hofmann et al., 2002; Van Oppen and Gates, 2006). Chaperones, such as heat shock elements, are involved in multiple functions including homeostatic regulation and response to a wide range of stressors (temperature, light,

pollutants, pathogens, etc.). This highlights the importance of using large suites of biomarkers, such as gene expression profiling with a focused microarray, to prioritize the classes of stressors impacting field populations.

Other environmental stressors

-salinity

Another environmental parameter that is seasonal and could have a strong impact on coral gene expression is precipitation. Heavy precipitation reduces salinity, increases terrestrial runoff, and overloads wastewater treatment facilities. Thus, precipitation events may expose corals to osmotic stress, sedimentation, xenobiotics, and/or increased nutrients, all of which have been implicated in declining coral reef health (Dubinsky and Stambler, 1996; Kerswell and Jones, 2003; McLaughlin et al., 2003; Philipp and Fabricius, 2003; Weber et al., 2006). During this study, large precipitation events occurred in Dade County immediately before the June (2005) and October collections (Figure 4.2b). Precipitation in June (2005) was the highest recorded during the period, followed by late September and early October (2005). In addition, the October collection occurred just after hurricane Wilma and tropical storm Ophelia hit the region. Our collection sites are likely to be differentially impacted by precipitation events due to their locations. Sites 1 and 2 are approximately 5 – 6 kilometers southeast of the Miami River outlet and the Virginia Key sewage outfall and east of the Biscayne Bay outlet. Site 3 is approximately 6 kilometers north of these inputs, while sites 4 and 5 are more than 25 kilometers north of the river, bay and outfall and are expected to be much more weakly impacted by these inputs. The Miami River and Biscayne Bay are significant sources of

freshwater, sediment and contaminants to nearby reefs (McArthur, 2001; Long et al., 2002; Gardinali et al., 2004).

Several studies have demonstrated that salinities below 30 ppt are stressful and potentially lethal to coral, in part because they have a limited capacity to osmoregulate (Ferrier-Pages et al., 1999; Kerswell and Jones, 2003). Decreased salinity affects metabolism, disrupts cellular processes and enzyme kinetics, negatively impacts photosynthesis and respiration, and causes decreases in reproduction and survivorship (Muthiga and Szmant, 1987; Moberg et al., 1997; Ferrier-Pages et al., 1999; Manzello and Lirman, 2003). Salinity from site 2 was measured as 30 ppt at the surface in June (2005) and local flooding was noted in the area on 20 June 2005 (personal observation, *unpublished*). Ambient salinities in the region range from 36 to 37 ppt at the surface and vary by less than 0.5 ppt from 0 to 60 m depth (DesRosiers, 2007). Several studies have demonstrated reduced photosynthesis and respiration, and even bleaching at salinities below 30 ppt (Kerswell and Jones, 2003; Manzello and Lirman, 2003). Ferrier-Pages et al., (1999) showed similar responses at salinities as high as 34 ppt. In this study, genes involved in cellular respiration and protein modification are significantly decreased in samples collected from sites 1 – 3 in June (2005) and October (Appendix, Figure A.9). In addition, genes involved in the regulation of apoptosis are decreased (Table 4.2). This latter response is especially significant because apoptotic regulation is thought to be important in maintaining the coral-algal symbiosis (Perez and Weis, 2006; Rodriguez-Lanetty et al., 2006). Other genes on the array that show increased expression are involved in proteolysis, DNA repair, oxidative stress and wound healing (Table 4.2 and

Appendix, Figure A.9). Of the symbiont-specific genes, all are elevated except a peridinin chlorophyll-a binding protein gene, photosystem II D1 (psbA) and an ultraviolet-B-inducible ribosomal protein gene. These genes show consistently decreased expression, indicating a decrease in photosynthesis. Our observations therefore are consistent with coral physiological responses to low salinity that have been demonstrated in other studies.

-*sedimentation*

Increased deposition of sediment and organic matter on coral reefs is another factor that could be associated with precipitation events. Sediments were observed on corals at site 2 on several collection dates. This site also exhibits lower species diversity than other sites, has few, if any, soft coral and is dominated by sediment tolerant scleractinian coral species, such as *M. cavernosa* (*personal observation*). Additionally, the US Army Corps of Engineers and Port of Miami began dredging the Miami harbor and channel on July 11, 2005 and dumping the processed sediments offshore (directly east of the Miami River inlet). Negative impacts of sediments on corals result from tissue abrasion, light reduction, smothering, energetic costs of removal, and exposure to pathogenic microbes, nutrients, and organic xenobiotics bound to the sediments (Rogers, 1990; McLaughlin et al., 2003; Nugues and Roberts, 2003; Weber et al., 2006). Studies investigating coral response to sediment stress have shown reduced chlorophyll-a and zooxanthellae concentrations, decreased photosynthesis and increased respiration (Riegl and Branch, 1995; Philipp and Fabricius, 2003; Weber et al., 2006). An induction of molecular chaperones, Hsp 70 and Hsp 90, has also been shown to occur in response to sediment

stress (Wiens et al., 2000; Hashimoto et al., 2004). One study demonstrated increased Hsp 70 expression in response to sediment stress and elevated temperatures, but not decreased salinity (Hashimoto et al., 2004).

The results of this study indicate decreased expression of genes involved in photosynthesis in highly impacted samples (June, 2005 and October sites 1 and 3) and several genes involved in wound healing show elevated expression. However, cellular respiration is decreased and molecular chaperones, including multiple Hsp 70 and Hsp90 genes, reveal either inconsistent or non-significant expression. In addition, a gene isolated in response to dark conditions (Edge et al., 2005) did not show consistent expression across these samples. The species we examined, *M. cavernosa*, has thick tissues, large polyps, and is generally found on reefs with high rates of sedimentation due to its ability to effectively clear sediments (Lasker, 1980; Flood et al., 2005). These observations suggest that although sediment stress may be a factor in the observed response, it is most likely not the primary stressor impacting the samples from sites 1 through 3 during the months of June and October (2005).

-effluent

Sewage from Dade County, and other regions of South Florida, is disposed of through multiple ocean outfalls (Caccia and Boyer, 2005). One of these is the Virginia Key sewage outfall. Nutrient enrichment from treated sewage has demonstrated direct and indirect impacts on coral in controlled laboratory and *in situ* experiments (Ferrier-Pages et al., 2000; Ward and Harrison, 2000; Harrison and Ward, 2001; Bruno et al., 2003;

Fabricius, 2005). However, scientific opinion is divided as to whether nutrients are directly harmful to coral health in the field (Szmant, 2002). Physiological responses by coral to direct exposures of excess nutrients include decreased reproduction (Harrison and Ward, 2001), calcification (Marubini and Atkinson, 1999), growth and photosynthesis (Ferrier-Pages et al., 2000). However, a cellular response to nutrient stress in coral has not been documented.

In other organisms biomarkers of exposure to sewage effluent focus on endocrine disrupting compounds (Diniz et al., 2005; Tyler et al., 2005; Hoger et al., 2006). For example, several studies have demonstrated increased production of vitellogenin in fish exposed to xenoestrogens found in sewage effluent (Rankouhi et al., 2002; Hoger et al., 2006; An et al., 2007). A vitellogenenin-like gene has been documented in, *Galaxea fascicularis*, a hermatypic coral (Hayakawa et al., 2005) and a vitellogenin gene from Hydra is present on our coral stress array. Our results indicate significantly decreased expression of vitellogenin in corals collected in June (2005) from sites 1 and 2, and October, site 2. Samples collected in October from sites 1 and 3, and June (2005) site 3 show little deviation from the StdLsmean of vitellogenin expression. In contrast, June (2005) sites 4 and 5, and October, site 4 show significantly elevated levels of expression (Appendix, Figures A.2 and A.9). Based on the studies cited above, this does not suggest exposure to endocrine disrupting compounds, which is an indicator of exposure to sewage effluent. However, it should be noted that little is known regarding the expression of vitellogenin in coral under normal physiological conditions or in response to stress. In addition, cnidarian hormonal signaling pathways are poorly characterized and endocrine

disruption has not been documented (Tarrant, 2007). Based on other physiological responses observed in coral exposed to elevated nutrients, it is hypothesized that genes involved in growth and development, respiration and photosynthesis should decrease in expression. Genes in these categories, however, are impacted by many exogenous stressors. Further work is needed regarding the effect of sewage effluent on coral gene expression to determine the mechanisms behind a cellular response. Coral gene expression responses to elevated nutrients, endocrine disrupting compounds, and lowered salinity from effluent exposure should be examined in controlled exposure experiments to isolate each effect.

-xenobiotics

It is well known that terrestrial run-off in developed areas exposes coral to xenobiotics such as fertilizers, pesticides, heavy metals, and polyaromatic hydrocarbons (Burke et al., 1998; McLaughlin et al., 2003; Fabricius, 2005). In addition, the Miami River is heavily polluted (Long et al., 2002), so it is likely that reefs adjacent to the outlet would be impacted by such contaminants. However, our results indicate a decreased level of expression of genes related to xenobiotic exposure at sites 1, 2 and 3 during June (2005) and October, no significant difference in June (2006) and August, and a distinct increase in the xenobiotic response in February at sites 1 and 2 (Table 4.2). One explanation for this observation is that the heavy precipitation may reduce the impact of xenobiotics either through dilution or changes in water movement. Another explanation involves the genes in the xenobiotic category. Specific molecular functions for some of the genes have not been characterized, but these genes were isolated from corals exposed in the

laboratory to heavy metals (copper, mercury), pesticides (dibrom, permethrin) or polyaromatic hydrocarbons (naphthalene) (Morgan et al., 2001; Morgan and Snell, 2002; Morgan and Snell, 2006).

-*synergistic stress*

Finally, a synergistic effect between all, or a few, of the stressors related to precipitation, such as lower salinity, sedimentation, sewage and xenobiotics, is another possibility for the observed response. Synergistic stress has been demonstrated in coral exposed to elevated temperatures during periods of high solar radiation (Jones, 1998; Lesser 2004). The combined stress causes greater damage to the photosynthetic complex of the symbionts, leading to a greater bleaching response at lower temperatures (Lesser 2004).

During precipitation events, several stressors can co-occur and be interrelated. For example, organic xenobiotics and heavy metals can bind to sediments from terrestrial run-off (Linzey et al., 2003, Morgan et al., 2005, Maenpaa et al., 2003) and may expose sediment-laden corals to xenobiotics for an extended period of time, potentially exacerbating the absorption of the compounds. In addition, corals are particularly sensitive to lipophilic contaminants because they possess thick, lipid-rich tissues that aid in the direct uptake of such compounds (Peters et al., 1997 – Tarrant 2007). Sewage effluent also contains xenobiotics and organic pollutants, including endocrine disruptors, pharmaceuticals (anti-inflammatory drugs, lipid regulators, antibiotics, etc.) and complex compounds like polybrominated diphenyl ethers (PBDEs) (Song et al., 2006b; Muller et al., 2007; Radjenovic et al., 2007). These pollutants are flushed into the ocean at

discharge sites, along with nutrients and fresh water. Depending on the distance of an outfall from a reef, horizontal and vertical mixing, and current speed, sewage outfalls can impact corals in multiple ways. Reduced salinity or exposure to xenobiotics may stress a coral, causing direct or indirect impacts of nutrients to be exacerbated. In addition, contaminants in sewage effluent have been shown to bind to sediments (Bubb and Lester, 1995; Mortimer and Connell, 1995; Dubinsky and Stambler, 1996), which may be deposited on corals, for instance after a storm. Future studies should characterize stress response gene expression patterns produced by exposure to multiple stressors acting synergistically.

Other expression patterns

-Site 4

Another pattern apparent in the results is a weak, but consistent elevation of genes in the stress response category at site 4 across all dates, except February. This points to a local or site-specific stress since it is present in all but one collection. However, it also could be related to temperature or light exposure since the pattern is only evident during the warmest months of the year (> 27 °C) and site 4 is a relatively shallow site (10 m). Increased exposure to light has direct and indirect effects on coral and is intimately linked to temperature stress (Fitt et al., 2001). Both exposure to ultraviolet radiation (UVR) and elevated temperatures have been shown to induce antioxidant enzymes and heat shock elements in coral (Downs et al., 2000a; Wiens et al., 2000; Brown et al., 2002a; b; Levy et al., 2006). In addition, the synergistic effect of these stressors causes DNA damage, apoptosis and decreased production of pigments, such as green fluorescent proteins, in

the host (Fitt et al., 2001; Lesser and Farrell, 2004; Dove et al., 2006). In symbionts, these stressors disrupt carbon dioxide fixation causing oxidative induced damage to the photosystem II complex and decreased production of D1 protein, which is responsible for photosynthetic electron transport (Jones et al., 1998; Warner et al., 1999; Lesser and Farrell, 2004; Perez and Weis, 2006).

Results in this study reveal that stress genes elevated above the StdLsmean expression at site 4 are involved in host pigmentation (green fluorescent protein gene), DNA repair (N-methylpurine DNA glycosylase), proteolysis (ubiquitin, RING-H2 zinc finger and polyubiquitin), apoptosis and inflammation (NALP/cyropyrin and cystatin) and protein folding (Hsp 70 and a glucose regulated protein gene). Additionally, a selenium binding protein gene is elevated, which has been implicated in oxidative stress (Song et al., 2006a) (Table 4.2 and Appendix, Figure A.2).

Xenobiotics do not appear to play a major role in the stress response observed on these dates at site 4, since all genes involved in a response to xenobiotic stress are either not significantly different or below the StdLsmean (Table 4.2 and Appendix, Figure A.9). However, the elevated expression of genes involved in DNA repair, inflammation, protein folding, proteolysis and photoprotective pigment production are consistent with the stress response associated with exposure to high light intensities in the host (Salih et al., 2000; Lesser and Farrell, 2004; Edge et al., 2005). Expression patterns of the symbiont genes do not show a similar relationship to light stress and genes involved in oxidative stress are not significantly different between samples. Exceptions include

catalase, which is elevated in both June samples (2005 and 2006) at site 4, but not in August or October, and the selenium binding gene which is elevated on all dates at site 4, except February (Table 4.2 and Appendix, Figure A.2). Based on these expression profiles and the relatively weak stress responses, it is difficult to diagnose a cause of stress at this site. It is important to note that significant increase or decrease of gene expression in a sample is relative to the level of expression in all other samples. Therefore, a dramatic response, such as that observed in samples from June (2005) and October, sites 1 and 3, may mute observed stress in other samples, making a specific identification more difficult.

-*February, site 2*

Although the overall stress response (SR) at site 2 in February is not significantly different than other group responses (NCF, MF and ZOOX, $p = 0.8292$), several genes related to xenobiotic exposure are significantly expressed above the StdLsmean ($p = 0.0190$, Table 4.2 and Appendix, Figure A.9). These include genes isolated in response to direct exposures to copper, mercury, dibrom, or permethrin (Morgan et al., 2001; Morgan and Snell, 2002; Morgan and Snell, 2006). In addition, ubiquitin, cytochrome b, ceruloplasmin and metallothionein are elevated. Two metabolic genes encoding acyl coenzyme-A thioesterases also reveal increased expression (Table 4.2). Acyl-CoA thioesterases have previously demonstrated increased expression in response to multiple xenobiotics (Tamura et al., 2006). These results suggest that corals at this site are experiencing stress from an unidentified contaminant in February.

Symbiont-Specific Responses

Another distinct pattern to emerge is a tight positive correlation between the expression of coral stress response genes and the symbiont-specific genes on the array (Figure 4.5). This response is consistent across all degrees of stress observed in the samples. In a stressed sample, such as coral from October site 1, elevated symbiont genes include actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribulose 1;5-bisphosphate carboxylase oxygenase (RuBisCO), subtlilisin/kexin isozyme, ferredoxin, peridinin chlorophyll-a, and a *Symbiodinium microadriaticum* calmodulin 1 gene (SMCaM1). In non-stressed samples, such as February site 4, these same genes are similarly decreased. Three of the zooxanthellae genes exhibit the reverse of this response. These include a peridinin chlorophyll-a binding protein gene, a UVB-inducible ribosomal protein gene, and the photosystem II protein D1 (psbA) gene. These three genes are significantly decreased in stressed samples and increased in samples that exhibit little stress gene expression (Table 4.2 and Appendix, Figure A.9).

These results are noteworthy for several reasons. First, it has been demonstrated that the photosystem II D1 protein decreases with increased exposure to light and elevated temperatures (Warner et al., 1999; Lesser and Farrell, 2004). In addition, changes in this protein are associated with damage to the photosystem II complex due to oxidative stress (Jones et al., 1998). Therefore it is significant that the gene encoding this protein exhibits markedly decreased expression in samples from our study that show elevated stress gene expression. Additionally, RuBisCO is the key photosynthetic enzyme that catalyzes the first step of CO_2 fixation. Oxidative stress in coral is caused, in part, by a disruption of

carbon dioxide fixation in the zooxanthellae (Jones et al., 1998). Damage to the photosystem II complex follows the resulting redox imbalance (Jones et al., 1998; Lesser and Farrell, 2004). This impairment in the ability of the symbionts to photosynthesize may represent a signal initiating the dissociation of the coral-algal symbiosis (Kerswell and Jones, 2003). In addition, the biological process associated with the SMCaM1 gene is ciliary or flagellar motility (UniProtKB/TrEMBL entry O15931; GO:0001539). Within the host, zooxanthellae are immobile and lack flagella. Outside the host, zooxanthellae acquire two flagella and are free-living, mobile dinoflagellates (Titlyanov and Titlyanova, 2002). The significant increase in expression of a flagella motility gene during stress may be a response by the zooxanthellae to signals indicating the breakdown of symbiosis. The algal symbiont may be preparing to leave a host that has become inhospitable. This requires further research, but may lead to very interesting results.

Conclusions

This study reveals that microarray technology can be used to compare levels of stress between coral populations in the field and detect responses to stress events at specific sites in South Florida. These stress events appear to correlate with periods of heavy precipitation in the region. In addition, a site experiencing xenobiotic stress during at least one of the collection dates was identified and a different site was shown to be experiencing mild stress over time. Observing and quantifying gene expression profiles produced by coral in the field narrows the list of potential stressors impacting populations and enables the generation of specific hypotheses regarding which stressors have the greatest impact on a population. For example, based on the results of this study, I

hypothesize that corals exposed to decreased levels of salinity will produce the same gene expression profiles as corals in the June (2005) and October (2005) samples from sites 1, 2 and 3. This hypothesis can be tested by experimentally exposing coral to decreased salinity and quantitatively comparing their gene expression profiles to those of field collected coral from this study. Future studies should also investigate coral gene expression patterns produced by manipulative experimental exposures to Miami River and sewage outfall effluents. Finally, the expression of the symbiont-specific genes on the array is shown to correlate strongly with the expression of host stress response genes. Further investigation is required to determine whether coral stress responses are regulating zooxanthellae gene expression and whether zooxanthellae prepare to exit coral at the onset of coral stress gene expression. This is important because it could provide valuable information regarding cellular and molecular mechanisms behind stress induced coral bleaching.

Concluding Remarks

Coral live in dynamic environments and are regularly challenged by a variety of stressors. Maintaining normal cellular and physiological functions in the face of stressful environmental conditions is fundamental to homeostasis and the continuity of life. In response to stress, corals alter the expression of a subset of genes to minimize or repair the impacts of stress. By tuning into these processes, gene expression profiling can be an especially informative method for understanding stress in corals. The advantages of gene expression profiling as compared to observing physiological responses to stress, lie in the ability to detect sublethal responses and reveal the cellular mechanisms underlying the response. My research has demonstrated that when incorporated onto a microarray, changes in the expression of suites of genetic markers can be used to identify classes of stressors impacting coral health.

Like any technology, gene expression profiling using microarray analysis has certain limitations. Results can be influenced by technological variability including differences between hybridizations, efficacy of reverse transcription, and DNA labeling efficiencies. Most of this variability is eliminated by proper experimental design and robust statistical analyses, such as log transformation, normalization and ANOVA. Biological variability and the complexity of biological systems may also influence results. Genetic heterogeneity, previous exposure to stress, the ability to reproduce sexually and asexually, and the colonial nature of coral can make interpreting a response difficult. In addition, gene expression within a single cell varies in complexity and activation with

genes exhibiting ranges in expression over time and under different environmental conditions. This variability is controlled by appropriate biological replication from within and between colonies. Finally, gene array technology does not consider the effect of stress on translation, post-translational modification, protein localization, or protein degradation. However, changes in gene expression are still interesting and relevant, especially if they represent reliable, sensitive, and selective markers of a response to specific conditions.

The research described in this dissertation demonstrates the feasibility of incorporating genomic tools, such as microarrays, into ecological field studies. Our results demonstrate the capacity of gene expression profiling in diagnosing coral health in natural populations and justifies future studies employing microarrays in coral research. Several additional studies using the coral stress microarray are in progress. For example, the coral microarray is being used to compare gene expression patterns between acute and chronic exposures of the same stressor and to identify the impacts of effluent exposure on gene expression. In addition, genes differentially expressed in response to infection by pathogens have been isolated. Once characterized, these genes will be incorporated onto the array to test the interactive effects of environmental stress and disease infection on coral. The incorporation of microarray based gene expression profiling into ever increasing environmental research projects is a testament to the usefulness of information produced by this technology.

APPENDIX

Table A.1. Comprehensive list of oligonuceotide sequences incorporated on the array, the corresponding probe, gene name, accession number, sequence, length, and melting temperature (TM). TBS indicates a sequence that is in the process of submission to GenBank and has not yet been assigned an accession number.

Probe	Gene Name	Accession	Sequence	bp	Tm
SpkCtrl: NAC1| AF198054_481_515	Arabidopsis thaliana NAC1	AF198054	tcgaggccgtaaaaccgattgggtcatgcacgaat	35	74.4
SpkCtrl: NAC1| AF198054_569_603	Arabidopsis thaliana NAC1	AF198054	gaagactgggtcttgtgtagggtattccataagaa	35	67.7
SpkCtrl: NAC1| AF198054_361_395	Arabidopsis thaliana NAC1	AF198054	aactaaccgagcaacggccaccggatattggaaag	35	73.2
SpkCtrl: NAC1| AF198054_1165_1199	Arabidopsis thaliana NAC1	AF198054	tcctcactcttgtacccacggtagattcatgtaaa	35	68.7
SpkCtrl: NAC1| AF198054_1059_1093	Arabidopsis thaliana NAC1	AF198054	gctgatgatcgagtgtaacgagagttactattgct	35	68.0
SpkCtrl: RCP1| AF168390_1208_1242	Arabidopsis thaliana root cap 1	AF168390	tggcttatggtcacaactcgccgtttagatctttg	35	70.4
SpkCtrl: RCP1| AF168390_1016_1050	Arabidopsis thaliana root cap 1	AF168390	ggcttatgatccctcgagcactatttatccgtgat	35	69.4
SpkCtrl: RCP1| AF168390_1144_1178	Arabidopsis thaliana root cap 1	AF168390	ttcttcgtggcagctacaattggtttgatctcatg	35	69.4
SpkCtrl: RCP1| AF168390_968_1002	Arabidopsis thaliana root cap 1	AF168390	taaaaggcttatcgtcaatcacaatgttgctctcg	35	67.9
SpkCtrl: RCP1| AF168390_704_738	Arabidopsis thaliana root cap 1	AF168390	gggaagacgttatcactattggtggactctccgtt	35	70.6
NC-SP-phage-03-7	phage	N/A	gacggtgatcgcatcggtgctgttaacaagttcat	35	72.0
NC-SP-phage-05-2	phage	N/A	ccatgattacgccagttgtacggacacgaactcat	35	71.5
NC-SP-phage-06-1	phage	N/A	gactctccggattaactggcggtgacggtaatttc	35	71.5
NC-SP-phage-08-9	phage	N/A	gtgaccataccgtacaggttagaaaccgtcagcgt	35	72.3
NC-SP-phage-09-4	phage	N/A	cgaccaaatcgttgtaaatccccgtaaaggcagat	35	70.5
NC-SP-phage-12-6	phage	N/A	gttacggttgatttcgagttgggtccacttatcgc	35	70.7
Dk(C)A2_17_51	Transferrin	AJ300650	ccgggcaggtacctgtcgctcatggttgttaaaat	35	73.2
Dk(C)A2_145_179	Transferrin	AJ300650	agcaacgcttattctagttccgtttctgcataggt	35	69.8
Dk(C)A2_89_123	Transferrin	AJ300650	gtgaccactgcatgcccgggattattgccaagatt	35	73.7
Dk(C)A2_113_147	Transferrin	AJ300650	ttgccaagattgcaactttcatgggctttgccagc	35	73.6
Dk(C)A2_44_80	Transferrin	AJ300650	gttaaaatcgtaatgatatcgccgatcttgttgttgc	37	67.6
Dk(C)F5_17_51	Scavenger receptor type A (SR-A)	XM_775770	ggccgaggtactatctggatggatgacgtcaagtg	35	72.2
Dk(C)F5_133_167	Scavenger receptor type A (SR-A)	XM_775770	tgtggagtgcgagtaaaaccattacagaattgtaa	35	67.3
Dk(C)F5_57_91	Scavenger receptor type A (SR-A)	XM_775770	gaggagaggcatcgctactactctgtgctcataat	35	70.2
Dk(C)F5_103_137	Scavenger receptor type A (SR-A)	XM_775770	tgagagctgtagtcatagcgaggatgcaagtgtgg	35	72.2
Dk(C)F5_203_240	Scavenger receptor type A (SR-A)	XM_775770	gcaataccaatcgacactacttatttgaaactgacacg	38	68.4
DkC(F1)_225_259	Ferritin	AJ306614	tgcagacaagcacaatgactaccagatggccgact	35	74.0
DkC(F1)_305_339	Ferritin	AJ306614	agatctccggacacatcaccaacctgaagcgtgtc	35	73.9
DkC(F1)_91_125	Ferritin	AJ306614	cgtggaggcctgttgaagctgaccgacatcaagtg	35	74.7
DkC(F1)_178_212	Ferritin	AJ306614	aagttggagaggcatgtgaacgagtccctgctgag	35	73.9
DkC(F1)_47_81	Ferritin	AJ306614	aggagctggagcacgccatgaagctgatgaagttc	35	74.4
DkC(G10)_81_115	unknown	N/A	cgcaagcttgacctatcggactaaatgcgcctcat	35	73.1
DkC(G10)_21_55	unknown	N/A	gaggtacatctcgagcccacttgtccgtccagaac	35	73.6
DkC(G10)_225_259	unknown	N/A	cagtgttccatgcagagatgtatttgtaggccttc	35	69.1
DkC(G10)_141_175	unknown	N/A	cgtagagtgccttgccctgagccgtctttccattc	35	74.5
DkC(G10)_260_294	unknown	N/A	ttgccagcagcattctgcaacttcgacacgacctt	35	74.9
DkC(G11)_17_51	Ly-6; uPAR	TBS	ccgggcaggtacaacatctaagaagtgcattgagt	35	71.5
DkC(G11)_209_243	Ly-6; uPAR	TBS	ctaaccttgcaagtcacgccgctatactcaccaca	35	72.7
DkC(G11)_94_128	Ly-6; uPAR	TBS	ggcttggttcgcggcttatatgagtagtgccaggc	35	74.7
DkC(G11)_161_195	Ly-6; uPAR	TBS	ttcaaggagacatgtgagcccacgttgcattcgtc	35	73.3
DkC(G11)_291_325	Ly-6; uPAR	TBS	ccaccaaggtcgcgctatccttcttgagaaacctc	35	72.9
SedB(H5)_177_211	28S rRNA	AB126711	tctaggtggatcggcggtgcctaagttgcttggaa	35	75.0
SedB(H5)_327_361	28S rRNA	AB126712	taaagctaaatactggcgtgagaccgatagcgaac	35	69.7
SedB(H5)_258_292	28S rRNA	AB126713	gctgacgatgtgctttcgaagagtcgggttgtttg	35	72.5
SedB(H5)_73_107	28S rRNA	AB126714	gaaactaacaaggattccctcagtaacggcgagtg	35	70.2
SedB(H5)_25_59	28S rRNA	AB126715	tggcaaggctacccgctgaatttaagcatattaat	35	69.0
SedC(A6)_129_163	28S rRNA	AY026375	tcatcgaccgacctattctattcttaggaaggttt	35	67.2
SedC(A6)_25_59	28S rRNA	AY026375	ggtctaacatgtgcgcgagtcttagggtgagtgaa	35	72.4
SedC(A6)_191_225	28S rRNA	AY026375	aaagatggtgaactatgcctgaatagggtgaagcc	35	70.1
SedC(A6)_340_374	28S rRNA	AY026375	ttccctcaggatagctggaactcagtacctcggcc	35	74.3
SedC(A6)_297_331	28S rRNA	AY026375	gaaagactaatcgaactgtctagtagctggttccc	35	68.0
SedC(C9)_1_17_51	Thioredoxin	AY652616	ggccgaggtcgttcgtgtgtcagattatctatctt	35	70.7
SedC(C9)_1_453_487	Thioredoxin	AY652616	tgcggtagtatgctatcgtttgctgtacctgcccg	35	74.0
SedC(C9)_1_97_131	Thioredoxin	AY652616	gagtgtgagactctcgcagccttcaacgaagagct	35	73.7
SedC(C9)_1_344_378	Thioredoxin	AY652616	gtggacactgtagtcggagctagtgaggccaaagt	35	73.4
SedC(C9)_1_205_239	Thioredoxin	AY652616	tcccaaaatatcagagttttcggaggtctacaccg	35	69.5
SedC(C9)_2_23_57	425 large subunit rRNA	AY064532	tatcggtctcacgccagtatttagctttagatgga	35	68.8
SedC(C9)_2_87_121	426 large subunit rRNA	AY064533	aaacaacccgactcttcgaaagcacatcgtcagcg	35	73.4
SedC(C9)_2_48_82	427 large subunit rRNA	AY064534	tttagatggagtttaccacccatttttgggctgcat	35	70.4
SedC(E9)_105_140	TRAPD	NM_001002082	tgcgacaataatgctaaggagttaaacttgtatgct	36	67.5
SedC(E9)_171_205	TRAPD	NM_001002082	aaatccacagatgatgcaaaattccaggtcagctg	35	69.7
SedC(E9)_28_63	TRAPD	NM_001002082	ccacaccagaaatctactccacaaggaacattttgc	36	69.8
SedC(E9)_131_165	TRAPD	NM_001002082	cttgtatgctgaagtgaatgggcgagttttacctg	35	69.5

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
SedC(E9)_149_183	TRAPD	NM_001002082	tgggcgagttttacctgtaacaaaatccacagatg	35	69.2
CX_cont(19c4)_155_189	peridinin chlorophyll-a binding	AY149154	gacgtattgttgcctcggtgccaaaagcgactgtg	35	74.1
CX_cont(19c4)_10_44	peridinin chlorophyll-a binding	AY149154	tttcacaagatgattgtcatgggtagcaagatgga	35	68.5
CX_cont(19c4)_185_219	peridinin chlorophyll-a binding	AY149154	ctgtgatggatgtctacaacttcattgccatgcat	35	69.4
CX_cont(19c4)_119_153	peridinin chlorophyll-a binding	AY149154	ctgctgcagactatgaggctgtgaacgcagctatt	35	73.0
CX_sal(19c4)_37_71	N-acetylglucosamine	AK222645	aggagactaatccatctacggtatcgaacactgtt	35	68.4
CX_sal(19c4)_81_115	N-acetylglucosamine	AK222645	ttgtttgatatccaccacgctgatccttcattgcc	35	70.8
CX_sal(19c4)_13_47	N-acetylglucosamine	AK222645	cacaagatgattgtcgtgttgtgaaggagactaat	35	67.4
CX_sal(19c4)_57_94	N-acetylglucosamine	AK222645	gtatcgaacactgttttgatggctttgtttgatatcca	38	68.4
Hsp27_cont(B1)_17_51	Hsp 27	NM_079276	gttcatggatggtcgcagttcatggatgtgtcgca	35	73.4
Hsp27_cont(B1)_69_103	Hsp 27	NM_079276	cagttcaaggagccaggtgatgtacaggaactccc	35	72.6
Hsp27_cont(B1)_41_75	Hsp 27	NM_079276	gatgtgtcgcagttccatggatgtgtcgcagttca	35	73.3
Hsp27_cont(B1)_89_123	Hsp 27	NM_079276	tgtacaggaactccctggactggaggattcattgc	35	72.2
Hsp27_cont(B)_113_147	methionylaminopeptidase	NM_180394	acaacagactaatcctcctacagtggcgatatcag	35	68.9
Hsp27_cont(B)_185_219	methionylaminopeptidase	NM_180394	catgagctacaaagacgacaatctgtggcgtgaaa	35	70.6
Hsp27_cont(B)_261_295	methionylaminopeptidase	NM_180394	atctgtagtgatgtgagacaagctgccgaggctca	35	73.3
Hsp27_cont(B)_313_347	methionylaminopeptidase	NM_180394	atgtgcaaggatttatcaagcctggattgactatg	35	67.7
Hsp27_cont(B)_153_187	methionylaminopeptidase	NM_180394	ttccccaatggcaactttcctatgggacaaatcat	35	70.4
Hsp27_T31(1)_11_45	regulator of nonsense transcripts	XM_417288	atgtgtcgcagttccaatgcaccttaagggacaga	35	72.7
Hsp27_T31(1)_152_186	regulator of nonsense transcripts	XM_417288	ctctggactggcagctaacatccgggacagacaga	35	74.6
Hsp27_T31(1)_221_257	regulator of nonsense transcripts	XM_417288	actggtacttgattataatcagagactggaggattca	37	67.2
Hsp27_T31(1)_41_75	regulator of nonsense transcripts	XM_417288	acagagggctcgtcacaatagtctgagtgaagaaa	35	70.4
Hsp27_T31(1)_121_155	regulator of nonsense transcripts	XM_417288	aacagtttaaagatctccagattcctctcagctct	35	67.5
SOD_A(A)_9_43	Cu/Zn superoxide dismutase	AY164664	cgcccttaatttgaaaggcttaccaccagaaggca	35	72.2
Hsp90(1)8_133_168	chromatin modifying protein 2A	NM_026885	cctgtaagctcgtcggtaagagtaagtccaagttca	36	70.5
Hsp90(1)8_9_43	chromatin modifying protein 2A	NM_026885	aatatcaagctgttattcccgtcgaagattttcca	35	67.2
Hsp90(1)8_361_395	chromatin modifying protein 2A	NM_026885	cctttcatcgcttgagccatggcattgttggatcg	35	72.9
Hsp90(1)8_263_298	chromatin modifying protein 2A	NM_026885	tgtccataatttcagactgtttctcaaactccatca	36	67.1
Hsp90(1)8_385_419	chromatin modifying protein 2A	NM_026885	ttgttggatcgcaatgtctggactttaagtgatac	35	67.6
Hsp90(2)3_61_95	NALP; zinc finger protein; Cryopyrin	XM_692820	cctcctcaggtctagtacgactagtttacatttgt	35	67.2
Hsp90(2)3_148_183	NALP; zinc finger protein; Cryopyrin	XM_692820	accactcagatatagcttggatagtttacagcttgg	36	68.0
Hsp90(2)3_17_51	NALP; zinc finger protein; Cryopyrin	XM_692820	agatttctgagagattgcgcactttcctcactcat	35	69.7
Hsp90(2)3_120_154	NALP; zinc finger protein; Cryopyrin	XM_692820	tggtaacaccggtattggtgatctgattaccactc	35	69.4
Hsp90(2)3_37_71	NALP; zinc finger protein; Cryopyrin	XM_692820	actttcctcactcataccataattcctcctcaggt	35	68.6
PU_cope(17A)_77_111	polyubiquitin	AF184280	atccagaaggagtccaccctccacttggtcctcag	35	74.4
PU_cope(17A)_6_40	polyubiquitin	AF184280	cactcaccggcaagcataagacactcaccggcaag	35	74.9
PU_cope(17A)_F_97_131	polyubiquitin	AF184280	ggaagaccattaccctcgaggtagagcccagcgac	35	74.8
PU_cope(17A)_F_41_75	polyubiquitin	AF184280	accatggtcctcagaactgcgaggtggtatgcaga	35	74.7
PU_cope(17A)_F_21_55	polyubiquitin	AF184280	cagaaaggagtccaccctccaccatggtcctcaga	35	74.5
PU_cope(17B)_74_108	Troponin T (TNT)	AF133521	gaggtcgtcagggcagagatgctcgaagcaatctg	35	74.1
PU_cope(17B)_133_167	Troponin T (TNT)	AF133521	caagaagcccaagagccgtctacccaagtggttcg	35	74.5
PU_cope(17B)_225_259	Troponin T (TNT)	AF133521	atgttgccggagaagcagcagttgaggaatacgat	35	72.8
PU_cope(17B)_21_55	Troponin T (TNT)	AF133521	gtaccgacaccaggacctacggcgacagaaagaag	35	73.7
PU_cope(17B)_45_79	Troponin T (TNT)	AF133521	acagaaagaagttgtacgagggaggctgggaggtc	35	73.3
GST_cont(10A)_273_307	dystonin isoform 1	XM_778863	caaaatcttcgaccttctcgtggtacttgtaaagg	35	67.7
GST_cont(10A)_185_219	dystonin isoform 1	XM_778863	tgctctagctgtttgactatcatcttagtgtcaca	35	67.5
GST_cont(10A)_45_79	dystonin isoform 1	XM_778863	acatcttggtcacgtactgtaactgcgcctccacg	35	74.0
GST_cont(10A)_95_130	dystonin isoform 1	XM_778863	ggctggcttgcaatcacttctacagcgatctccttc	36	73.3
GST_cont(10A)_138_173	dystonin isoform 1	XM_778863	caaaatccggcttgtggttctcgtaatctgtcatca	36	70.5
Dbs_193_227	Dibrome	BI534456	agagtcataggaagcttgccttgtattcattgcca	35	69.9
Dbs_345_379	Dibrome	BI534456	gttcagatgtaactagtgtccatataccagcggag	35	68.0
Dbs_269_303	Dibrome	BI534456	gtatgtggctagcaagtagatatggcaagtggtgg	35	70.1
Dbs_9_46	Dibrome	BI534456	catgttgatagagacacttctaaactgccttatgtctt	38	67.2
Dbs_87_125	Dibrome	BI534456	tgatcaatgttttaaagtcctcctaccaaatactgaaca	39	67.3
PM_9_43	Permethrin	XM_928237	aggccaatgacataggttgtcttcttagcttacca	35	69.3
PM_109_143	Permethrin	XM_928237	tccaactgctgtatacatcagagatcagtttcctt	35	67.7
PM_69_103	Permethrin	XM_928237	ctgagtacaaacgccagaaggcagattatcaccaa	35	70.2
PM_27_61	Permethrin	XM_928237	gtcttcttagcttaccatcctaagcgcaacatcat	35	68.7
PM_87_121	Permethrin	XM_928237	aggcagattatcaccaaaatatttccaactgctgta	35	67.2
CU_81_115	Copper	BI534458	gcagctatgcgaaacactaaggaactgtaagtttc	35	68.2
CU_21_55	Copper	BI534458	tcttggaatattcaattgacatgagggcagatgga	35	68.3
CU_105_140	Copper	BI534458	ctgtaagtttctagaacggaaacatcccttaacaca	36	67.2
Hg_53_87	Mercury	BI534459	aatatgcagtattaatgttaagcagcgaggaagca	35	67.5
Hg_125_159	Mercury	BI534459	cgctggcgtgaagattttatgaatgttgcataccc	35	70.2
Hg_171_208	Mercury	BI534459	tgttttaaatgcaacgcaatttgtaccttgcttcaaat	38	68.4
Hg_23_62	Mercury	BI534459	tctaaattcatcgctgacataaaagagtgtaatatgcagt	40	67.5
Hg_71_105	Mercury	BI534459	taagcagcgaggaagcatttcggtttcaattttta	35	68.3
Dbl_113_147	Dibrome	BI534457	cgtcgtccctcatagctgcgcaaataagcatttct	35	72.0

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
Dbl_425_459	Dibrome	BI534457	ggtaacatcgctggtgtcgactggcgtataatgaa	35	71.3
Dbl_269_303	Dibrome	BI534457	catccacaaaacgtcataatttatgccgttgtcaa	35	67.3
Dbl_153_188	Dibrome	BI534457	tagtaaaacgagagatttgagatctggtgcaagacg	36	68.4
Dbl_199_235	Dibrome	BI534457	atcattttgaagtcgatttccatgcatacaaagacag	37	67.2
Np_25_59	Ceruloplasmin (naphthalene)	DN167139	cagaccttcacttatcggtggactgtgccggagga	35	74.7
Np_285_319	Ceruloplasmin (naphthalene)	DN167139	gaaagccaatgcgagtcttataaacgacggtatga	35	68.8
Np_97_131	Ceruloplasmin (naphthalene)	DN167139	tgggtgtatcactccagtgttgatcctgttaagga	35	70.2
Np_325_359	Ceruloplasmin (naphthalene)	DN167139	tctgttacaaacgctatggcatttatgctgtcctg	35	69.3
Np_145_179	Ceruloplasmin (naphthalene)	DN167139	ttatttggtccactgcttacatgcaagaagggaac	35	69.3
C+2C27_9_43	Copper	TBS	gtgctttaccctggaaaccggccttaactatagcg	35	71.8
C+2C27_93_127	Copper	TBS	ggcctggttcttgacttgaagacacgaaccgtttg	35	72.5
C+2C27_75_109	Copper	TBS	tgcctgcacatgactgaaggcctggttcttgactt	35	74.4
C-5G28_150_184	Copper	TBS	tttaatgaatatcctggcgtccatcagcatggcaa	35	70.4
C-5G28_21_55	Copper	TBS	gcaaacattttccatagcattagggagacaggctc	35	69.5
C-5G28_47_83	Copper	TBS	gacaggctcctttgtctaatattgagggaacataatt	37	67.3
C-5G28_97_131	Copper	TBS	gcacacttacatgagcaatatgtgaggtctttctt	35	67.7
P+3G22_105_139	Permethrin	TBS	attttggcaacacattgactggaatcttcatccta	35	67.6
P+3G22_3_37	Permethrin	TBS	tttggtcagggtaatgtccttattaacatggaagg	35	67.2
P+3G22_42_77	Permethrin	TBS	agtgaagacccaattttaggagaatgtatttcacca	36	67.0
P+3G22_61_99	Permethrin	TBS	gagaatgtatttcaccagtcttttctctttgaaaggaac	39	67.1
MGID_9_209_243	regulation of translation initiation	NM_199820	tgtcgatctccgaagaagtcatattgctaaggagg	35	69.1
MGID_9_281_316	regulation of translation initiation	NM_199820	cattgtatcacttgaggatgtcattcgttacttcct	36	67.1
MGID_9_103_137	regulation of translation initiation	NM_199820	aagaaccagctttggatgcattgtatgatgtcatt	35	68.0
MGID_9_257_291	regulation of translation initiation	NM_199820	caagttgatctgccagcaagttaacattgtatcac	35	67.2
MGID_9_41_75	regulation of translation initiation	NM_199820	ttactttcagcgaccggaaaatgctctgaaaagag	35	69.3
MGID_10_93_127	peptidyl-prolyl cis-trans isomerase	XP_424751	tccaagaatggctattacgacgatatcatcttcca	35	67.6
MGID_10_209_243	peptidyl-prolyl cis-trans isomerase	XP_424751	agttcgaggatgatttccaccggaacttgaagcac	35	71.7
MGID_10_53_87	peptidyl-prolyl cis-trans isomerase	XP_424751	ccaggagtgtccgaagtcggtggagaatttcacgg	35	74.1
MGID_10_441_476	peptidyl-prolyl cis-trans isomerase	XP_424751	gtccgatccaaaatcagtgaccattcaaaatcatca	36	68.1
MGID_10_129_163	peptidyl-prolyl cis-trans isomerase	XP_424751	cgagtcattcagggcttcatgatccagacgggtga	35	73.7
MGID_12_385_419	peroxiredoxin	XP_422283	agatttggtatgtggcaacggaggattcaacaata	35	68.7
MGID_12_5_39	peroxiredoxin	XP_422283	aagaaccggcttccagcgtctgttcaactacattt	35	71.7
MGID_12_507_541	peroxiredoxin	XP_422283	tcttcttgacaatgcgtgtctcgatcgtttgtgaa	35	70.2
MGID_12_65_99	peroxiredoxin	XP_422283	gatgcagatgacggctcctgttcgggaacgcattt	35	74.8
MGID_12_297_331	peroxiredoxin	XP_422283	cttcagtcgaaagggattgatgtcaatgcagacag	35	69.3
MGID_19_417_451	NADH dehydrogenase (ubiquinone)	NP_998304	gatggcagtcgtcgaacaacacgctatgacattga	35	71.8
MGID_19_133_167	NADH dehydrogenase (ubiquinone)	NP_998304	aggcacatgaggtaccaatggattggcatagttcc	35	71.6
MGID_19_41_75	NADH dehydrogenase (ubiquinone)	NP_998304	gcagcacggaatgattccaaaaccgcattctctga	35	72.5
MGID_19_337_371	NADH dehydrogenase (ubiquinone)	NP_998304	aagagaggtgtattgcttgcaaattatgtgaagcc	35	68.2
MGID_19_245_280	NADH dehydrogenase (ubiquinone)	NP_998304	ggaaccagcaacaattaattatccatttgagaaggg	36	67.2
MGID_22_113_147	cathepsin B	AAQ83887	tgcatgaaccactcactaaagaagcgattgactac	35	68.8
MGID_22_19_53	cathepsin B	AAQ83887	gctagctgttaacagtgctaatattgttgcagaac	35	67.0
MGID_22_217_251	cathepsin B	AAQ83887	aagagtttgtgcggcgtcccgttagatggcaagat	35	74.4
MGID_22_538_572	cathepsin B	AAQ83887	gaagctgcatggaattactgggttgactctgggct	35	73.0
MGID_22_369_403	cathepsin B	AAQ83887	aatccgtgaccaagggtcttgtggaagttgctggg	35	74.5
MGID_30_119_153	RuBisCO	AF298221	aggcactgtgagagtaaagtgtcttgcccaagaac	35	71.5
MGID_30_295_329	RuBisCO	AF298221	atacttaattagtggcatttacaatggcaccagct	35	67.5
MGID_30_319_353	RuBisCO	AF298221	tggcaccagcttggggttcagaatttgtacatgtg	35	72.3
MGID_30_41_75	RuBisCO	AF298221	gtatgggatgctagtccatcgcagggttaccccca	35	74.8
MGID_30_73_107	RuBisCO	AF298221	ccatcattaagttcgccggtacccatttatacacc	35	69.0
MGID_47_194_229	Ferredoxin	P10770	gattctgcctacttgtgtgacctacgccactttctg	36	71.6
MGID_47_73_107	Ferredoxin	P10770	gcttggagttgccatattcttgccgagctggctct	35	74.7
MGID_47_139_173	Ferredoxin	P10770	gcagcattgaccagagcgaccaggcattctttgga	35	74.9
MGID_47_321_355	Ferredoxin	P10770	ggacctaatgccaaagtgccacacttacagaacag	35	70.8
MGID_47_237_271	Ferredoxin	P10770	catcaaagacccactgtgaggacgagctctgagca	35	73.7
MGID_62_161_195	Ferrochelatase	AAK16728	caatgtcagacctcatcgaaaccgaaatggcgggc	35	73.9
MGID_62_249_283	Ferrochelatase	AAK16728	gagttatgtggaactggctggtgatccatacaagg	35	70.1
MGID_62_293_328	Ferrochelatase	AAK16728	aggagtgtgtggaccttattatgaatgagctgcaga	36	70.6
MGID_62_111_146	Ferrochelatase	AAK16728	gaagcacacagtcataccatcctggtaccagcgacc	36	74.0
MGID_62_205_239	Ferrochelatase	AAK16728	actgaaggtgtggagatattcttctctgctcatgg	35	69.3
MGID_68_273_307	PABPC4 - regulation of translation	XM_417821	gctggagattgacaatatccgagctgttgcacatgt	35	72.0
MGID_68_409_443	PABPC4 - regulation of translation	XM_417821	gtgcccgtgtaactaagattcacttctaccggaca	35	70.8
MGID_68_113_147	PABPC4 - regulation of translation	XM_417821	aggtgcagcagaccattcaaatacctggacaggat	35	72.5
MGID_68_49_83	PABPC4 - regulation of translation	XM_417821	cagaatgtacgtaaccagcctgcacaaggacatcc	35	71.9
MGID_68_201_235	PABPC4 - regulation of translation	XM_417821	gttaggtgaacgcttgttccccatgatccaagcca	35	73.3
MGID_83_163_197	oligosaccharyl transferase	EAA12890	tgctaacagggatcattcaattcgtctactgttgt	35	68.3
MGID_83_273_307	oligosaccharyl transferase	EAA12890	tgtctacgagttcagagtaatcctgccaacatgtc	35	69.4
MGID_83_377_411	oligosaccharyl transferase	EAA12890	ggttgtcatgaatttcattggctaacatggatgca	35	68.6

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
MGID_83_83_117	oligosaccharyl transferase	EAA12890	cctggtgaaattctacgatgaatacaaccagacga	35	68.4
MGID_83_313_347	oligosaccharyl transferase	EAA12890	tcaagggaatctcaccagagagagcatttgccgac	35	72.6
MGID_94_153_187	glucose regulated protein	AAH44524	aggaaccaagtactggcgcaaccgtgttatgaaag	35	72.0
MGID_94_389_423	glucose regulated protein	AAH44524	atggtgctcttaaaccttacatcaagtctgaacca	35	68.3
MGID_94_225_259	glucose regulated protein	AAH44524	aattgcagccaaacaggactattcaaacgactttg	35	68.7
MGID_94_457_491	glucose regulated protein	AAH44524	gttgttggtgagactttcaatgacattgtcaaccg	35	68.8
MGID_94_503_538	glucose regulated protein	AAH44524	gatgttttaatccgagtttctatgcaccttggtgtg	36	68.4
MGID_113_277_311	Hsp 90	CAC38753	gtttgacggacccgacgaggcttgacagcactaaa	35	74.3
MGID_113_17_51	Hsp 90	CAC38753	aaacgccgcaccgacgttatccttgctactttgct	35	74.7
MGID_113_145_179	Hsp 90	CAC38753	ttcgcttttcaagccgagatcgctcagttgatgag	35	71.9
MGID_113_301_335	Hsp 90	CAC38753	acagcactaaagaacttaagattgagcttatcccc	35	67.1
MGID_113_331_365	Hsp 90	CAC38753	tccccaacagatatgacaacaccttgacgatcatc	35	69.8
MGID_114_199_233	glucose regulated protein	CAA53369	gtcgctcttgaaggactacttcggcggcaaggaac	35	74.5
MGID_114_513_547	glucose regulated protein	CAA53369	ggacaaccaccttctcggcaaagttcgaactcacc	35	73.4
MGID_114_431_466	glucose regulated protein	CAA53369	tcacaagacctttccacctaccaaggacaaccagc	36	72.8
MGID_114_5_39	glucose regulated protein	CAA53369	gagatcgagggcttgtacgacggcgtcgacttctc	35	74.9
MGID_114_51_85	glucose regulated protein	CAA53369	cgcgcgcgcgttttgaagagttgaacttggacttg	35	74.7
MGID_135_142_176	Hsp 90	AAP51222	gaggctcctttactgttcgccgtcttgcagaggag	35	74.1
MGID_135_65_99	Hsp 90	AAP51222	ctacttggttgctgacaaggttgaagtttacacca	35	69.1
MGID_135_205_239	Hsp 90	AAP51222	ttcacctcaaggaagatcagcaggaatatttggag	35	68.4
MGID_135_8_42	Hsp 90	AAP51222	gcaggctggtgccgatatctctatgattggtcagt	35	72.4
MGID_135_252_287	Hsp 90	AAP51222	aaagagattgtaaagaagcacagccagttcattggt	36	69.2
MGID_192_377_411	glutathione-s-transferase	AAT09082	aactctcgccaagttcgtaggagctgatccctttct	35	73.0
MGID_192_5_39	glutathione-s-transferase	AAT09082	aaggcgttgataaacttactctatgttctcgacgg	35	68.3
MGID_192_217_251	glutathione-s-transferase	AAT09082	acccaccaaactagtccaggatcaaatcggaggaa	35	71.9
MGID_192_473_507	glutathione-s-transferase	AAT09082	caaatatctggcatccaacttgtacaagctaccct	35	68.7
MGID_192_121_155	glutathione-s-transferase	AAT09082	ggtcatcgcttgtgaggaattgttatgtgaaatca	35	68.0
MGID_213_47_81	peridinin chlorophyll-a	L13613	ggcaaaggtcatggacgtctacaatgccgtgaagg	35	73.6
MGID_213_502_536	peridinin chlorophyll-a	L13613	ctatcggacgcattgttgcatcggtgccaaaagcg	35	74.5
MGID_213_111_145	peridinin chlorophyll-a	L13613	atgaagtctttggtcaatggtgcggacgcggagaa	35	74.3
MGID_213_287_321	peridinin chlorophyll-a	L13613	gaaagaggtggactggctgtctgacgtttacctga	35	72.3
MGID_213_354_388	peridinin chlorophyll-a	L13613	gtgaaagccatcgacaagatgatcgtgatgggcag	35	72.2
MGID_235_145_179	aldehyde dehydrogenase	XP_419732	agaggttattaagcgagctaacggtgtccagtatg	35	69.3
MGID_235_209_243	aldehyde dehydrogenase	XP_419732	ctttcacgaacccaccgagtgtcaaaacgcttaca	35	72.1
MGID_235_49_83	aldehyde dehydrogenase	XP_419732	gacccacggttgtgactggtttgtccgatagcact	35	74.1
MGID_235_381_415	aldehyde dehydrogenase	XP_419732	tcaagatgtagggtccacgacagtcatgtcatttt	35	69.5
MGID_235_281_315	aldehyde dehydrogenase	XP_419732	gacctgaatatgccgttcggaggattcaaggcttc	35	71.8
MGID_264_181_216	Ferritin	AY456681	ctcttcaacctgatgggctatagagtttaacatctg	36	67.0
MGID_264_128_162	Ferritin	AY456681	aagctgaccttgcaagatgacgactagaagctgtc	35	71.3
MGID_264_2_36	Ferritin	AY456681	cctcagatgatggatttcattgagggaaatttcct	35	67.4
MGID_264_83_118	Ferritin	AY456681	aacttgaagcgtgttggccctggactgggtgaatac	36	74.5
MGID_264_405_439	Ferritin	AY456681	catttgcccaagtgtgtcaaagaggttgaacatta	35	68.4
MGID_289_17_51	selenium binding protein	NP_543168	gagttgcgttatctgacccaatattagcaagccac	35	69.4
MGID_289_281_315	selenium binding protein	NP_543168	caaaggtcattcatcgtctgccagtaccttacaag	35	69.0
MGID_289_221_255	selenium binding protein	NP_543168	ggattgacaaaccagactaccttgccactgttgat	35	70.4
MGID_289_177_211	selenium binding protein	NP_543168	cagggagactctagtctaccttccatgtatccgta	35	69.1
MGID_289_505_539	selenium binding protein	NP_543168	ggacttggctatcttcatacaacacattgcctggc	35	70.9
MGID_293_49_83	Actin	BAC44869	gttctgttaaccgaggctccactcaaccctaaagc	35	71.7
MGID_293_171_205	Actin	BAC44869	cgcctctggccgtacaactggaatcgtctttgaca	35	74.2
MGID_293_219_253	Actin	BAC44869	cagtcacactgttcccatctatgagggttatgctc	35	69.8
MGID_293_279_313	Actin	BAC44869	cttggccggcagggacttgactgactacctcatga	35	75.0
MGID_293_110_145	Actin	BAC44869	tgtttgagaccttcaattcaccagccatgtacgtcg	36	71.8
MGID_307_245_279	RuBisCO	AF298221	atcagcttgagtgacggagggattgagtatgccaa	35	72.2
MGID_307_449_483	RuBisCO	AF298221	cagaaggatgctccgttcatgtgcgaaggctggtt	35	74.7
MGID_307_81_115	RuBisCO	AF298221	cgcttcgcttgccagcattcttcgagaatctggga	35	74.6
MGID_307_363_397	RuBisCO	AF298221	ctacactggtgagtcctccgacaggcggcatcttt	35	75.0
MGID_307_573_607	RuBisCO	AF298221	gagctaaaggaagatatctatgcttgagggccctt	35	69.3
MGID_344_365_399	alpha-tubulin 3	CAA76133	attgttgccatactgcatcacctaattgacatcct	35	68.8
MGID_344_325_359	alpha-tubulin 3	CAA76133	ggatttgcaccagttggcgttacaccatgttctgc	35	73.1
MGID_344_406_444	alpha-tubulin 3	CAA76133	ccaattgatattggtaatacttcacgaaccatcttgact	39	67.7
MGID_344_297_331	alpha-tubulin 3	CAA76133	tctaaatgaaccatcatctgcaggatctggatttg	35	67.3
MGID_344_345_379	alpha-tubulin 3	CAA76133	tacaccatgttctgcacaatattgttgccatactg	35	68.0
MGID_348_449_483	calcium ion binding	S51239	acaagtatgatgcattggagccattgggtttgatc	35	69.6
MGID_348_97_131	calcium ion binding	S51239	gctctaaggttgagagtggtgatcttgaatcagac	35	68.4
MGID_348_17_51	calcium ion binding	S51239	cagatgcaaggacgatgaatttacacatctctaca	35	67.2
MGID_348_57_91	calcium ion binding	S51239	attgtgaagcctgataacacttacgaggtgcgtat	35	69.7
MGID_348_316_350	calcium ion binding	S51239	ggagcctcccatgattgataaccctgactacaagg	35	71.0
MGID_373_177_211	small heat shock protein 16 - 48a	AAA28067	cttggtatcggagccccgcacttggtacaagttac	35	73.1

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
MGID_373_473_507	small heat shock protein 16 - 48a	AAA28067	aggaatattgatcgcgactctttcaagtccgctct	35	70.8
MGID_373_331_365	small heat shock protein 16 - 48a	AAA28067	ttcgtttgatgtgtccaaattcaagccagaggaga	35	70.2
MGID_373_97_131	small heat shock protein 16 - 48a	AAA28067	gcaccgaattaggcataatcctgaattttggtcag	35	68.4
MGID_373_417_451	small heat shock protein 16 - 48a	AAA28067	taaggaacgaagaaggcttttccaatcgccacttt	35	70.5
MGID_379_513_547	beta-1 tubulin (algae)	K03281	gagtcaccgagccgaatccaacatgaacgacctcg	35	74.3
MGID_379_457_491	beta-1 tubulin (algae)	K03281	tgtcaaggaggaaggctttcttgcactgggacact	35	73.9
MGID_379_33_67	beta-1 tubulin (algae)	K03281	ctcaacgctgacctgcgcaagttggctgtaaacct	35	75.0
MGID_379_167_201	beta-1 tubulin (algae)	K03281	tcagcagatgtgggacgccaagaacatgatgtgcg	35	74.8
MGID_379_257_291	beta-1 tubulin (algae)	K03281	catgtcctccaaagaaggggacgagcagatgctca	35	73.9
MGID_549_453_487	catalase - oxidoreductase activity	AAT06156	actatgtacatccaggtcatgacgtttgcacaggc	35	71.6
MGID_549_605_639	catalase - oxidoreductase activity	AAT06156	acagattgagccacgagcgccttttaaccctgttg	35	73.6
MGID_549_532_567	catalase - oxidoreductase activity	AAT06156	cacattctgattatccgctgatacaagttggaaaga	36	67.5
MGID_549_225_259	catalase - oxidoreductase activity	AAT06156	atcgtggaattcctgacggttatcagcacatgaat	35	70.0
MGID_549_299_333	catalase - oxidoreductase activity	AAT06156	ggagaggctgtctactgcaagttccatctcaagac	35	71.1
MGID_578_81_115	glyceraldehyde-3-phosphatedehydrogenase	NM_008085	ggcctggtacgacaatgagtatggctacagtcacc	35	72.5
MGID_578_217_251	glyceraldehyde-3-phosphatedehydrogenase	NM_008085	tcagtgtatggtatactccacttgttagcactgtg	35	67.9
MGID_578_37_71	glyceraldehyde-3-phosphatedehydrogenase	NM_008085	gctggtgctggtattgcccttaatcccaacttctt	35	72.0
MGID_578_169_205	glyceraldehyde-3-phosphatedehydrogenase	NM_008085	gagaagtcacagactcgtagctcactataatagatgt	37	67.1
MGID_578_9_43	glyceraldehyde-3-phosphatedehydrogenase	NM_008085	cggtgacactcactcgtgcatctttgatgctggtg	35	73.5
MGID_585_289_323	myosin heavy chain	CAA37311	agatcaatctagacaacgccctaaaggctaacgca	35	70.8
MGID_585_428_462	myosin heavy chain	CAA37311	caaagctgaacgccgtgtcggtgagatcaactcgg	35	74.8
MGID_585_193_227	myosin heavy chain	CAA37311	tggagagtctgcaagcatccttggaaaccgagatt	35	72.5
MGID_585_57_91	myosin heavy chain	CAA37311	gctgctctagaagccgaagaatccaaggtaacgcg	35	73.1
MGID_585_369_403	myosin heavy chain	CAA37311	ctgcaaatgatggttgaagaggaacaacgtggtcg	35	71.3
MGID_630_161_195	succinate dehydrogenase	XM_419054	ctctatggacacgtctctcaagtacgacatgtgct	35	70.4
MGID_630_17_51	succinate dehydrogenase	XM_419054	atgggatgccattcagtcgtacagaagacggtaaa	35	70.8
MGID_630_137_171	succinate dehydrogenase	XM_419054	caggtcactcactgttgcagcactctctatggaca	35	72.3
MGID_630_113_147	succinate dehydrogenase	XM_419054	gatgttgctgtgttgctgacagaacaggtcactca	35	71.9
MGID_630_37_71	succinate dehydrogenase	XM_419054	acagaagacggtaaaatctaccagagggcgtttgg	35	71.2
MGID_897_41_75	deoxyribonucleotide metabolism	NP_571525	gcttggtgtagacaactatatagtcaagagcgaca	35	67.9
MGID_897_501_535	deoxyribonucleotide metabolism	NP_571525	tccaaatagccaattgacgaaatatccactctgac	35	67.4
MGID_897_349_383	deoxyribonucleotide metabolism	NP_571525	atgactgggttaaactttcggatgatgaacaggta	35	68.2
MGID_897_217_251	deoxyribonucleotide metabolism	NP_571525	agcctcttcttcgtgaaaatccacaccgatttgtc	35	70.6
MGID_897_545_580	deoxyribonucleotide metabolism	NP_571525	gtctaccttgattcgaagacttacattgaggacttc	36	67.0
MGID_902_238_276	ultraviolet-B-inducible ribosomal (plant)	AY065657	gagtgcgatttaaggttgtaaaggttgctaatgtatcac	39	68.4
MGID_902_311_348	ultraviolet-B-inducible ribosomal (plant)	AY065657	tcgatcttaatgagactgtaacagcaataaacttgtgt	38	67.3
MGID_902_98_132	ultraviolet-B-inducible ribosomal (plant)	AY065657	gaggtacagctcattaagaatggcaagaagatcac	35	67.5
MGID_902_221_255	ultraviolet-B-inducible ribosomal (plant)	AY065657	tgttggtgatattcctggagtgcgatttaaggttg	35	69.0
MGID_902_141_175	ultraviolet-B-inducible ribosomal (plant)	AY065657	gtgccaaatgatggttgccttaatttcattgaaga	35	67.5
MGID_991_465_499	heat shock cognate 71	AAB21658	tcatgattccattagcatcaacgtcgaatgtgacc	35	69.1
MGID_991_101_135	heat shock cognate 71	AAB21658	gtaatgatgggactgcatacctttttcaacctcttt	35	67.6
MGID_991_163_197	heat shock cognate 71	AAB21658	agcagattcgttcttctctagccagtcaatgactt	35	69.3
MGID_991_279_313	heat shock cognate 71	AAB21658	tgttgtaagcataacttttccagggtattcttggcc	35	69.0
MGID_991_360_394	heat shock cognate 71	AAB21658	catctttcaccatgcgttctatgtcttccttggac	35	69.2
MGID_1003_159_193	Hsp 22	CA970652	agctttgttgccacacgagagatccgttgacatca	35	72.9
MGID_1003_257_291	Hsp 22	CA970652	gcgcggatgaaactgccaactagtgatggagactt	35	73.1
MGID_1003_133_167	Hsp 22	CA970652	ctgaccatagaagcatcacgtgatccagctttgtt	35	70.3
MGID_1003_67_101	Hsp 22	CA970652	gcaggtgtgctgccaaaagacgtgattctttccac	35	73.0
MGID_1003_284_318	Hsp 22	CA970652	ggagactttgacaagagcgaaattagctgaaactt	35	67.9
AY181557_577_611	green fluorescent protein	AY181557	gtggccattaccgatgtgactttcgaagtacttac	35	69.0
AY181557_713_747	green fluorescent protein	AY181557	gaatgccgaggctcgctattctatgctgccgagtc	35	74.3
AY181557_617_651	green fluorescent protein	AY181557	gaagaaacgtgttcagttgccagactatcactttg	35	68.3
AY181557_121_155	green fluorescent protein	AY181557	tggaaggcgctgtaaacgggcacaacttcgtgatt	35	74.8
AY181557_193_227	green fluorescent protein	AY181557	agactataaaccttacagtcaaagaaggcggacct	35	68.9
U60604_253_287	beta tubulin	U60604	cttgtctctgctacaatgagcggtgtaacaacctg	35	70.5
U60604_525_559	beta tubulin	U60604	tgttcgaacaaacgttagctatatgtcgccatgga	35	69.9
U60604_793_827	beta tubulin	U60604	gcgagtaacagctgaactggcggattaaaggtcaa	35	71.8
U60604_313_347	beta tubulin	U60604	caaagtaccgattcaaatccactgccgacttttgt	35	69.6
U60604_123_157	beta tubulin	U60604	cgctacgctgtccgttcatcagttggttgaaaaca	35	72.2
AF013738_105_139	cytochrome oxidase subunit I (cox I)	AF013738	gtgccaggcgcgatgttaggtgatgatcatcttta	35	71.6
AF013738_333_367	cytochrome oxidase subunit I (cox I)	AF013738	gaacaaggcgcaggaacgggatgaacggtttatcc	35	73.7
AF013738_575_609	cytochrome oxidase subunit I (cox I)	AF013738	gcctgtgttagcgggtgcaattactatgttattaa	35	67.7
AF013738_57_93	cytochrome oxidase subunit I (cox I)	AF013738	ggtctaattgggactgcttttagtatgcttatacgat	37	67.1
AF013738_455_493	cytochrome oxidase subunit I (cox I)	AF013738	aggagcaataaacttttattacaacgattttcaacatgcg	39	67.8
AB117374_173_207	mitochondrial cytochrome b	AB117374	cgtgtcttgcgtttacttctattgggcacataatg	35	68.4
AB117374_605_639	mitochondrial cytochrome b	AB117374	atgggtcaaataatcctatcggcttaaactcttcg	35	67.1
AB117374_779_813	mitochondrial cytochrome b	AB117374	ctttagttactcctgttcacattcaacccgagtgg	35	68.8

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
AB117374_999_1033	mitochondrial cytochrome b	AB117374	tttgacttgaattgggtcgcaagtagttgaagagc	35	69.4
AB117374_492_526	mitochondrial cytochrome b	AB117374	aggtgggtttagtgtttctggggctacattaaatc	35	68.7
L11067_3277_3311	Histone	L11067	tcggtataagtaacggcatcacggatgacattctc	35	69.5
L11067_3357_3391	Histone	L11067	cgagatgcgtttaacacctcctcgacgagctaggc	35	73.9
L11067_3485_3519	Histone	L11067	tgccacgtccggacatgttagctaattagacgttc	35	71.3
L11067_3205_3239	Histone	L11067	tacaaggtacgtccctggcgtttgagagcgtaaac	35	72.6
L11067_2837_2871	Histone	L11067	aacgagaatgcagttaacgtgaatattgcagaggt	35	68.9
AY532062_836_870	zinc finger transcription factor (snail)	AY532062	ctctcctcgttaaacaccagtattcttgcggaaat	35	68.7
AY532062_552_586	zinc finger transcription factor (snail)	AY532062	tgcctgcaagcattgcgacaagaactatctttcat	35	70.8
AY532062_423_457	zinc finger transcription factor (snail)	AY532062	taaaatctcacggaacgacgtcgactcgccaggaa	35	73.4
AY532062_748_782	zinc finger transcription factor (snail)	AY532062	cgttcgaatcttcgcgcacacctgcaaactcattc	35	72.9
AY532062_616_650	zinc finger transcription factor (snail)	AY532062	cacacgctgccatgcaagtgcactatttgtggaaa	35	73.1
AF285166_2534_2568	decapentaplegic (dpp)	AF285166	agagacaacatataggatcttcttagtgggcttga	35	67.1
AF285166_3030_3064	decapentaplegic (dpp)	AF285166	atcgacagtctctagggtagagtgactaactcagt	35	68.8
AF285166_2745_2781	decapentaplegic (dpp)	AF285166	tatgaattgacttactaattccctaacgttcatggcg	37	67.4
AF285166_2590_2624	decapentaplegic (dpp)	AF285166	tcgctgtttgaatcgttcctttggcgtagaaaacg	35	71.2
AF285166_2676_2710	decapentaplegic (dpp)	AF285166	catgtgaacaattcttgcgccacgaagtcagtagg	35	71.1
AF323688_1068_1102	nuclear receptor AmNR8	AF323688	ttcgccattaattggtgccgtaaggattgagaata	35	68.7
AF323688_1006_1041	nuclear receptor AmNR8	AF323688	attcttctccgtctgcctgctacacgaatgctgacc	36	74.0
AF323688_1326_1361	nuclear receptor AmNR8	AF323688	gcatgataagaaccacgatgacaatgccaacgatac	36	69.8
AF323688_1179_1216	nuclear receptor AmNR8	AF323688	aatactagtacaactaacttagttatcgagacgacggt	38	67.0
AF323688_1756_1792	nuclear receptor AmNR8	AF323688	tgtacataaatgactcagagattagaaagcttggcat	37	67.0
AF245689_494_528	homeodomain protein (cnox-2)	AF245689	gattgcagcgttgttagatctttccgagaagcaag	35	69.7
AF245689_270_304	homeodomain protein (cnox-2)	AF245689	aaggctgaacacgctcatttaagcccgcactatcc	35	73.3
AF245689_766_800	homeodomain protein (cnox-2)	AF245689	aaatggccgcgcgtgttaatcggatcagtaaatct	35	71.9
AF245689_326_360	homeodomain protein (cnox-2)	AF245689	gcgtttagtgtcatacgtttctcgtgccgatccgg	35	73.3
AF245689_927_961	homeodomain protein (cnox-2)	AF245689	tgtaaatacaccgagctgtaattattccgtttcgt	35	67.1
AB048853_171_206	Vasa-related protein CnVAS1	AB048853	aaactattgtcaagacagttgtggtatatggaggtg	36	67.1
AB048853_257_291	Vasa-related protein CnVAS1	AB048853	gtggcaacaccagggcgtctaaaggactttattga	35	71.8
AB048853_131_165	Vasa-related protein CnVAS1	AB048853	gcctgtcagatttacaatgaagctcgcaagttttc	35	68.9
AB048853_33_67	Vasa-related protein CnVAS1	AB048853	tgatgacaggaatgctgcagaaaggtttgaccagc	35	72.3
AB048853_89_123	Vasa-related protein CnVAS1	AB048853	cagtgtcctcaagctctaattatttcaccaactcg	35	67.6
AB048846_213_247	PL10-related protein CnPL10	AB048846	gtggagcagatattggcagtcagttgaaggaactt	35	70.2
AB048846_168_203	PL10-related protein CnPL10	AB048846	gaaagttttccaaccgttcttatgtgcgcccttgtg	36	71.8
AB048846_293_327	PL10-related protein CnPL10	AB048846	gatatgctggacagaggacgtgttgggctggacat	35	73.9
AB048846_13_47	PL10-related protein CnPL10	AB048846	tgctgccttcctcatcccaattttgagcagaatat	35	70.3
AB048846_237_271	PL10-related protein CnPL10	AB048846	tgaaggaacttgatcggggctgtcatctcctggtg	35	73.7
AF005356_2261_2295	integrin subunit betaCn1	AF005356	gaggctcccgtgttacctattgtacttggagttgt	35	70.8
AF005356_2133_2167	integrin subunit betaCn1	AF005356	aaggactttatcgttgcgagggaattgatggacaa	35	70.1
AF005356_1973_2007	integrin subunit betaCn1	AF005356	tgtcccaattgtgagaacggtatgtgtacacggaa	35	71.0
AF005356_2173_2207	integrin subunit betaCn1	AF005356	ttgtacttattactttaccaccgaaacggaggcag	35	68.5
AF005356_1785_1819	integrin subunit betaCn1	AF005356	aagacaagtatcatggcgacgcatgtgatcagaag	35	70.3
AF507185_81_115	Calmodulin	AF507185	taccaatagaactcagtttgcatgcattagtggct	35	68.7
AF507185_15_52	Calmodulin	AF507185	acaagataatgacagtggggaataatgattagtttgct	38	67.4
AF507185_99_133	Calmodulin	AF507185	tgcatgcattagtggctaaactgtacacagtgtat	35	68.6
AF507185_261_295	Calmodulin	AF507185	aaaactgaatgtaagggctggtccacttttattga	35	67.6
AF507185_150_188	Calmodulin	AF507185	tgtatatattcaacagtacatctttatttggtgggcagg	39	67.5
D30747_5239_5273	Mini-collagen	D30747	tgagccaatcgtaataagttcacaagcgcgggtaa	35	71.5
D30747_4415_4449	Mini-collagen	D30747	aaccgcttcacaatacagagtttccttggacaggt	35	71.3
D30747_5341_5375	Mini-collagen	D30747	gatggaaggccgtgttacatcgtgaacatcatcac	35	70.7
D30747_4496_4530	Mini-collagen	D30747	tgaatttcagttaggcgtgcaaccagtaaaatctg	35	68.2
D30747_5165_5199	Mini-collagen	D30747	acagtggaaacactctcggcgtagttgactgtggt	35	73.6
DQ351254_329_363	NADH dehydrogenase	DQ351254	gcctgcatatatcctaaaaccttttcgttcggctaa	35	68.9
DQ351254_370_404	NADH dehydrogenase	DQ351254	ataagctactgtgataagcaatggaactacgacca	35	67.8
DQ351254_491_526	NADH dehydrogenase	DQ351254	gtctcggaggttccaataatcaagacattctaagtc	36	67.1
DQ351254_689_723	NADH dehydrogenase	DQ351254	ttcaactattcaaagtcctcccagcatacagtgac	35	68.4
DQ351254_58_92	NADH dehydrogenase	DQ351254	ccacccagacattaatatcgcatagacacttatcg	35	67.1
AF152004_4757_4791	Hsp 70	AF152004	cacccacgtgtacgaacttgtagttgctatttcag	35	69.2
AF152004_4437_4471	Hsp 70	AF152004	aagtagagcgtgagtataagcgtgactgggcgtga	35	73.1
AF152004_4693_4727	Hsp 70	AF152004	tttcgcttgtagtgtcacggtgacttacgatgtaa	35	69.6
AF152004_4921_4955	Hsp 70	AF152004	gaaagttcggaccctatatatgcggctttaacact	35	68.5
AF152004_5144_5178	Hsp 70	AF152004	tgagtttaagggagtttgatgctagattgtacccg	35	68.3
AB201749_2139_2173	Hsp 70	AB201749	tcaaatcacggactagagtagctgatcgagtaaca	35	68.4
AB201749_1557_1591	Hsp 70	AB201749	atgccgacgggatcttaactgtttccgccaaggat	35	74.1
AB201749_1469_1503	Hsp 70	AB201749	gacaacaatctactgggacgatttgatctgaaagg	35	67.6
AB201749_1369_1403	Hsp 70	AB201749	gattccagcgcaagtttctaaggaagagttctcaa	35	68.8
AB201749_1905_1939	Hsp 70	AB201749	gagatctacagcaagtgtgttcaccagtcatgtcc	35	70.1
AY360081_1147_1181	Actin	AY360081	tgatgaatctggcccatccatcgttcgccgcaaat	35	74.8

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
AY360081_1055_1089	Actin	AY360081	cctcctgagcgtaagtactctgtatggatcggagg	35	71.4
AY360081_792_826	Actin	AY360081	atggacaggttatcaccatcggaaacgagagattc	35	69.6
AY360081_1360_1396	Actin	AY360081	tgaaaattagcagcaatacgacttcattactctctgt	37	67.2
AY360081_1451_1485	Actin	AY360081	gaaaataggcagccaatacgacttcatcattgcct	35	69.4
AB086183_864_898	Galaxin	AB086183	caaccaatcgtcttccttatgctgcggagctaccg	35	73.2
AB086183_704_738	Galaxin	AB086183	ctacccaattatgctgcgactctaacgtcttgccc	35	71.7
AB086183_1000_1034	Galaxin	AB086183	cttacaagtccaacttgctgcggaggagccgtgtt	35	74.6
AB086183_1508_1542	Galaxin	AB086183	gtttgagtttaggagttactgctaatcaacggagt	35	67.1
AB086183_1206_1240	Galaxin	AB086183	atgacacataagcatgtatagtcgtgcagtgtaac	35	67.3
AJ251054_257_291	F16P protien	AJ251054	gctaccatggttgttctaagcactggtaatggtgt	35	70.2
AJ251054_577_611	F16P protien	AJ251054	tctaatgtacgaatgcaatccaatggcgttcatag	35	67.7
AJ251054_673_707	F16P protien	AJ251054	aatccacgaacgcgcaccgattgctattggatcaa	35	73.4
AJ251054_473_507	F16P protien	AJ251054	gcccgctacgtcggctctatggtatctgacatgca	35	75.0
AJ251054_356_390	F16P protien	AJ251054	aaaccgcgaggtaagatttacagcatcaatgaagg	35	69.3
Y17848_1623_1657	Hsp 90	Y17848	gtttcgaagtactctacatgaccgaacccattgat	35	68.4
Y17848_2135_2169	Hsp 90	Y17848	gctcgtatttaccgaatgatcaacttgggtcttgg	35	69.2
Y17848_1575_1609	Hsp 90	Y17848	aggatcaggtctctaactcggctttcgtggagcgt	35	74.3
Y17848_1855_1889	Hsp 90	Y17848	ggtttcatcgccatgttgcatcgttacgagccagt	35	73.7
Y17848_2354_2388	Hsp 90	Y17848	agacatcgtgtcgtaattagaatcttgcaattcgt	35	67.2
U27840_54_88	Cu/Zn superoxide dismutase	U27840	cagagaggcatgtaggagatctgggcaatattgat	35	69.3
U27840_228_262	Cu/Zn superoxide dismutase	U27840	ttaccacaggtcacgctggaggtcgacttggttgt	35	74.9
U27840_117_151	Cu/Zn superoxide dismutase	U27840	acatacaggacagtcttgtgaagttaagtggagac	35	68.0
U27840_5_39	Cu/Zn superoxide dismutase	U27840	ggtcctcatttcaatccattcggtaagaaccatgg	35	69.2
U27840_179_214	Cu/Zn superoxide dismutase	U27840	gtacatgaaggtgtagatgatctgggcaagggaggc	36	72.5
AY164663_25_59	Cu/Zn superoxide dismutase	AY164663	agtccagtgatctttcgtactatctccgtgaattt	35	67.6
AY164663_87_121	Cu/Zn superoxide dismutase	AY164663	gctgcttgataggagaagttaagggaaccatcagc	35	70.3
AY164663_287_321	Cu/Zn superoxide dismutase	AY164663	gacgacactgaacgacatgtaggtgatctgggtaa	35	70.6
AY164663_661_696	Cu/Zn superoxide dismutase	AY164663	tgccacctaaattgcaccaatttaagctaaatttcg	36	67.7
AY164663_753_787	Cu/Zn superoxide dismutase	AY164663	aaatttgctagtgtaaccaggcctttagagttgat	35	67.2
AY164664_769_803	Cu/Zn superoxide dismutase	AY164664	attcactaagtgactaagattcccgctctcaaacg	35	68.4
AY164664_33_67	Cu/Zn superoxide dismutase	AY164664	caagcgtgtctgatcttagcatcacctaggacagt	35	70.7
AY164664_585_619	Cu/Zn superoxide dismutase	AY164664	gtttaaagactggtaacgctggagcacgtctggca	35	73.1
AY164664_713_747	Cu/Zn superoxide dismutase	AY164664	ttcagttcgcgatgctttagagtgatatgagatgg	35	68.4
AY164664_453_487	Cu/Zn superoxide dismutase	AY164664	gcagaatcaagataatgttgtccgactacttggtg	35	67.8
DQ309550_189_223	Cu/Zn superoxide dismutase	DQ309550	accagtatggagataataccaatggttgcaccagt	35	69.4
DQ309550_67_101	Cu/Zn superoxide dismutase	DQ309550	gtgttgcttggtaggagacgttaagggaaccatca	35	71.3
DQ309550_267_301	Cu/Zn superoxide dismutase	DQ309550	ctgacgacactgatagacatgtaggtgatatgggt	35	68.8
DQ309550_427_461	Cu/Zn superoxide dismutase	DQ309550	caaaggtggtcatgacgacagtcttacaactggcc	35	71.9
DQ309550_583_618	Cu/Zn superoxide dismutase	DQ309550	tgttttagagtcaacatacatgcatttgaatctggc	36	67.0
AY500892_1344_1378	Hsp 60 (mitochondrial)	AY500892	tggtggagtagcgttactcagacgtactaataact	35	68.3
AY500892_1297_1331	Hsp 60 (mitochondrial)	AY500892	gctttgaatgctacccgtgctgcagtagaagaagg	35	72.2
AY500892_792_826	Hsp 60 (mitochondrial)	AY500892	acaaatagtccctgctctagaacttgctaattccc	35	68.2
AY500892_1572_1606	Hsp 60 (mitochondrial)	AY500892	agaaggcatcatcgaccctaccaaggtggtacgta	35	72.8
AY500892_1204_1238	Hsp 60 (mitochondrial)	AY500892	agattagctaagctgtctgatggtgttgctatctt	35	67.9
DQ309544_33_67	calcium/calmodulin-dependent kinase IV	DQ309544	caaacagtacctcgactgacttacttccaaggatc	35	68.3
DQ309544_111_145	calcium/calmodulin-dependent kinase IV	DQ309544	agaacttctacgacataggaagagagctcggccgt	35	72.6
DQ309544_324_362	calcium/calmodulin-dependent kinase IV	DQ309544	tgtttatgatacttgaactagtaactggaggagaactgt	39	67.5
DQ309544_149_183	calcium/calmodulin-dependent kinase IV	DQ309544	gcttcatccgttgtcaagctctgccgacaaaaggg	35	74.0
DQ309544_377_411	calcium/calmodulin-dependent kinase IV	DQ309544	gagaagggtattatagtgaaaaggatgccgcaga	35	69.5
DQ309546_65_99	collagen alpha-1 chain	DQ309546	tttgtgataaccgacggagtacaaacacgcaggga	35	72.5
DQ309546_489_523	collagen alpha-1 chain	DQ309546	gtggatgacgtcataagcagtctcatcaatggatt	35	68.7
DQ309546_134_168	collagen alpha-1 chain	DQ309546	ctaaaagctaaaggtgtcagagtgtatgcgttggg	35	69.2
DQ309546_764_800	collagen alpha-1 chain	DQ309546	ccagccatcaatctaaccaacatatattcgtgaaaga	37	67.5
DQ309546_633_667	collagen alpha-1 chain	DQ309546	aatttgctgccattcgagccgaacgacgatggttt	35	73.8
DQ309539_353_387	acyl-CoA thioesterase	DQ309539	gaaagggactccggttaaacaagcgtgacgtttct	35	71.8
DQ309539_265_299	acyl-CoA thioesterase	DQ309539	gttatggacgatgtctgtcatggtggaacatatac	35	67.2
DQ309539_169_203	acyl-CoA thioesterase	DQ309539	gctgaagtgaccttacgagccaaacttactagtga	35	69.5
DQ309539_473_507	acyl-CoA thioesterase	DQ309539	attttatgggtaaaggcgtccgtaggattcctgta	35	69.2
DQ309539_69_103	acyl-CoA thioesterase	DQ309539	ggagacaacgaaggcctccgagccacaagtaatgg	35	74.4
DQ309537_217_255	S-acyl fatty acid synthetase thioester hydrolase	DQ309537	atggatattagtgtcagagttctaagagctgatcttact	39	67.1
DQ309537_73_107	S-acyl fatty acid synthetase thioester hydrolase	DQ309537	tctcatctcctaatatctggtgccgcaggacctga	35	72.6
DQ309537_121_155	S-acyl fatty acid synthetase thioester hydrolase	DQ309537	tcgcctggtgcatctcagttatctaatgaacagct	35	70.9
DQ309537_325_359	S-acyl fatty acid synthetase thioester hydrolase	DQ309537	tttgatggtagcaccgatcttcgtcagggagaagg	35	72.2
DQ309537_2_36	S-acyl fatty acid synthetase thioester hydrolase	DQ309537	gtcatagtctaggtgctgggatcgcctttgaagtg	35	71.3
DQ309536_113_147	ATP synthase (mitochondrial)	DQ309536	gctagaggtgatatgggtcgttttagagccactta	35	69.1
DQ309536_73_107	ATP synthase (mitochondrial)	DQ309536	cccagattgggtcaagttaagtacacgtgtaccta	35	69.2
DQ309536_286_320	ATP synthase (mitochondrial)	DQ309536	tccaaaggataccaagtctgatctgattgctaaga	35	67.6
DQ309536_193_227	ATP synthase (mitochondrial)	DQ309536	agccattgactgggagttttatgccaagaacgtct	35	71.5

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
DQ309536_377_412	ATP synthase (mitochondrial)	DQ309536	atcaaggagtatgaagcagagcttggtcaagttaaa	36	68.4
DQ309525_41_75	Cd36/Scavenger receptor class B	DQ309525	gaacacaacctatgcgaacatgctcaatgggagtg	35	71.1
DQ309525_301_335	Cd36/Scavenger receptor class B	DQ309525	ttggtgtgtgtcaagatgcaccgatatcactccca	35	72.4
DQ309525_493_527	Cd36/Scavenger receptor class B	DQ309525	ctctcattgagccagtcaaggacattgaacaaact	35	69.1
DQ309525_129_163	Cd36/Scavenger receptor class B	DQ309525	gttacccagctctgccgttcattatacctgacgta	35	70.8
DQ309525_569_604	Cd36/Scavenger receptor class B	DQ309525	tgagacagctaccattgataaaagtcaggcacaaat	36	68.7
DQ309521_129_163	plasma glutamate carboxypeptidase	DQ309521	actgggttagaggaaacgagtcagccactatgcta	35	71.5
DQ309521_641_675	plasma glutamate carboxypeptidase	DQ309521	caggttgtgttggtcagcggtcacctagatagctg	35	72.8
DQ309521_477_511	plasma glutamate carboxypeptidase	DQ309521	aaatcccaactgcctgtattacaatcgaagatgct	35	68.7
DQ309521_5_40	plasma glutamate carboxypeptidase	DQ309521	actgatacatttggcaatagaatatcagggtcgaag	36	67.1
DQ309521_583_617	plasma glutamate carboxypeptidase	DQ309521	ccctcctgttacgtccagaaacacggtggctgaga	35	74.9
AY836663_73_107	glutathione peroxidase	AY836663	atgcctcttaacctcggagatgttatgccaaactt	35	69.8
AY836663_217_251	glutathione peroxidase	AY836663	ttaggaagagttgagcagttgtcatccgaatttga	35	68.4
AY836663_367_401	glutathione peroxidase	AY836663	gctgacccaaaccgtgatattgcaatggcacttgg	35	73.1
AY836663_428_463	glutathione peroxidase	AY836663	agaaaggattgcctcttacttgcagagcggttttca	36	71.6
AY836663_305_339	glutathione peroxidase	AY836663	agggttggattgaagatatcaaggcatacaatggt	35	68.4
AJ251055_318_352	DHSB protien	AJ251055	gaatacattagcttgtatctgtcgcattgacggtg	35	67.9
AJ251055_190_224	DHSB protien	AJ251055	gatagctgtggtcctatggtactcgatgctttgat	35	69.3
AJ251055_734_768	DHSB protien	AJ251055	tgaactgtacaatgacatgccctaagggtctgaat	35	69.5
AJ251055_238_272	DHSB protien	AJ251055	gaaattgatccgacgctaacatttcgaagatcatg	35	67.0
AJ251055_566_600	DHSB protien	AJ251055	gttgtacaacgtcctgtccaagctattggtggcat	35	71.9
AY531316_135_169	arginine kinase	AY531316	tcattttaagccacgtgtcatccgagtagaattag	35	67.1
AY531316_181_216	arginine kinase	AY531316	cgagacattatgatatcattacgttttagcaggcgt	36	67.5
AY531316_57_91	arginine kinase	AY531316	atggagtcaagaaactactggagattgagcaagca	35	69.6
AY531316_336_371	arginine kinase	AY531316	gggatttctctaagatgcatcacattgagtgaaagt	36	67.5
AY531316_275_309	arginine kinase	AY531316	ttccttggaatgggattatatccgattcacggcga	35	70.8
AY841903_1596_1630	protein-tyrosine kinase (FAK)	AY841903	attataatggagcttgcagtttatggagagttgcg	35	67.8
AY841903_1782_1816	protein-tyrosine kinase (FAK)	AY841903	actgtgaagttagcagattttggactatcaaggtg	35	67.3
AY841903_1259_1294	protein-tyrosine kinase (FAK)	AY841903	tggagatttcgctgactatgcagaaattgatgataa	36	67.2
AY841903_1554_1590	protein-tyrosine kinase (FAK)	AY841903	atcatcaaattgattggaatatgtcctgaaaacccac	37	67.0
AY841903_1944_1981	protein-tyrosine kinase (FAK)	AY841903	tgctgggaaatacttatgtatggagtaaaacctttca	38	67.3
DQ218058_1155_1189	Caspase	DQ218058	gacaactgatcttctaaccatgatgacacgtgtga	35	68.5
DQ218058_963_997	Caspase	DQ218058	ccaaggtcatgaatacatggatggtgttgacgcaa	35	70.7
DQ218058_659_693	Caspase	DQ218058	tgcatcggtatcctcgaaatggaactgatgttgac	35	70.2
DQ218058_1099_1133	Caspase	DQ218058	gtgaatggtcatggtttatccagtccattgctga	35	70.4
DQ218058_1015_1049	Caspase	DQ218058	gacaagcgtgtccagatccctgtagaagcagactt	35	72.3
AY149139_641_675	peridinin chlorophyll-a binding protein	AY149139	tgcaaaagcgttgtccgatgcatcctatcctttca	35	71.9
AY149139_497_531	peridinin chlorophyll-a binding protein	AY149139	ttacatgaagtccttggtgaacgggcccgatgctg	35	74.5
AY149139_435_469	peridinin chlorophyll-a binding protein	AY149139	aaagcgaaggtcatggccgttacgattcagtgaa	35	71.8
AY149139_241_275	peridinin chlorophyll-a binding protein	AY149139	agcccttggaggcgttgaaagcaattgacaagatg	35	72.6
AY149139_686_720	peridinin chlorophyll-a binding protein	AY149139	cgattggctgtccgacatttatctgaagccgctgc	35	73.8
AB106689_273_307	glyceraldehyde-3-phosphate dehydrogenase	AB106689	ttgaatcgactggtctcttcgttgaggcagacaaa	35	71.1
AB106689_81_115	glyceraldehyde-3-phosphate dehydrogenase	AB106689	atatgtccaccgatgccgagtactttgcgtaccaa	35	72.4
AB106689_129_163	glyceraldehyde-3-phosphate dehydrogenase	AB106689	ccgttcatggccgcttcaagcacgatgtcaagatc	35	73.9
AB106689_785_819	glyceraldehyde-3-phosphate dehydrogenase	AB106689	aaggcgaccgagacctacatgaagggcgttctctc	35	74.8
AB106689_433_467	glyceraldehyde-3-phosphate dehydrogenase	AB106689	gtccaatgcctcttgcaccaccaattgcttggcac	35	74.8
AB086828_381_415	Actin (algae)	AB086828	ggatagacttggccggtcgcgatctcacggagtac	35	74.8
AB086828_545_579	Actin (algae)	AB086828	acatcctccgaccttgagaagacatacgagctgcc	35	73.4
AB086828_653_687	Actin (algae)	AB086828	ggcaaagaggcgtcgggaatccatgagactgtgtt	35	74.4
AB086828_604_638	Actin (algae)	AB086828	gaatgagcgcttccggtgtccagaagtgctgttcc	35	74.9
AB086828_713_747	Actin (algae)	AB086828	gacattcgcaaggatctctttggcaacgtggttct	35	72.1
AF007889_481_515	Symbiodinium calmodulin (SMCaM1)	AF007889	cgcaatttccggctcgaagctgtatcatggatcgc	35	73.8
AF007889_357_391	Symbiodinium calmodulin (SMCaM1)	AF007889	tgtggatggtgacggccagatcaactatgaggagt	35	73.2
AF007889_223_257	Symbiodinium calmodulin (SMCaM1)	AF007889	ctcatcgaggcattcaaggtcttcgaccgcgatgg	35	74.4
AF007889_115_150	Symbiodinium calmodulin (SMCaM1)	AF007889	caggacatgatcaacgaggtcgacgccgatggaaac	36	74.4
AF007889_435_469	Symbiodinium calmodulin (SMCaM1)	AF007889	cgcctgcatgtaaagatcactggccgtcactgatg	35	73.5
AJ884906_137_171	psbA gene for photosystem II protein D1	AJ884906	atcttaatgcacccattccatatcctaggagtagc	35	67.8
AJ884906_185_219	psbA gene for photosystem II protein D1	AJ884906	ggatcattctttagtgcaatgcatgggtcattagt	35	67.8
AJ884906_252_291	psbA gene for photosystem II protein D1	AJ884906	acatttcactcaatcttggatataagtttggtcaagaaga	40	67.5
AJ884906_39_73	psbA gene for photosystem II protein D1	AJ884906	ttgtttacccgattggtcaagctagcttctcagat	35	69.6
AJ884906_91_125	psbA gene for photosystem II protein D1	AJ884906	aagtggaacattcaatttcatgcttgtcttccaag	35	67.2
AF299359_543_577	RuBisCO	AF299359	ccctggtgtactacatcgaccctgatagcgaggag	35	72.9
AF299359_401_435	RuBisCO	AF299359	aagcacgtgctggtggcatacatcatgaagccgaa	35	74.7
AF299359_3_37	RuBisCO	AF299359	agacgacctctgccctctgctcaatcagaatgacg	35	73.6
AF299359_500_534	RuBisCO	AF299359	gtgaacgtgtgcaccaccgacgacttcacgaagtc	35	74.7
AF299359_325_359	RuBisCO	AF299359	gacacgcaaggcattggaccagtccagccgatatg	35	74.9
DR681654_2_36	Metallothionein	DR681654	gcccttgtaattgcattgagatagctccttgtaat	35	67.7
DR681654_137_175	Metallothionein	DR681654	gcaagtgtaaatatcaacgttctactaaatgtgactcca	39	67.7

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
DR681654_73_107	Metallothionein	DR681654	cgcaaaaacgtcccaacatttatacttgttagggg	35	68.6
DR681654_25_59	Metallothionein	DR681654	gctccttgtaattgcattgagctccacagcgtgga	35	73.5
DR681654_99_133	Metallothionein	DR681654	tgttaggggtatttttgcgttatgtgagcctgcaa	35	70.6
DY447310_589_623	multidrug resistance protein-1	DY447310	gcaagacgtaggatggtttgatgttaatgaacctg	35	68.1
DY447310_412_448	multidrug resistance protein-1	DY447310	tgttggaagggataagcaatatgacatattaggttcc	37	67.2
DY447310_534_569	multidrug resistance protein-1	DY447310	ctgcttcacgtcaggttcataaaattcgaatgaaat	36	67.1
DY447310_337_371	multidrug resistance protein-1	DY447310	tgcatctattggaaatggggttgctcaacctgcct	35	73.2
DY447310_614_648	multidrug resistance protein-1	DY447310	aatgaacctggaacattgacaacaagactttcaga	35	67.6
DT622365_50_84	multidrug resistance efflux pump	DT622365	caggcggattttcaccggaaaccgctggggaatat	35	74.8
DN252355_233_267	multidrug resistance efflux pump	DT622365	ggagtaggatctagcacgccacaagtagaatccac	35	70.8
DN252355_305_339	multidrug resistance efflux pump	DT622365	cttttgacatctactgcgtctgctagtgaaacaac	35	68.1
DN252355_358_392	multidrug resistance efflux pump	DT622365	aacagctgtttcgtctcaacctgatattgaaactt	35	67.6
DN252355_109_146	multidrug resistance efflux pump	DT622365	ttcgggtaaaattcttgatgatgataaagctttatgcg	38	67.2
DN252355_333_368	multidrug resistance efflux pump	DT622365	aaacaacttcagtatccactatctcaacagctgttt	36	67.2
DT608217_593_627	cathepsin B	DT608217	aaaggcattgttactggtggtccatacaattctca	35	68.7
DT608217_329_363	cathepsin B	DT608217	gtgcacgaccttcctattgagtttgatgctcgtaa	35	70.2
DT608217_233_270	cathepsin B	DT608217	ggccctcatataacttcagactacattaggaatttgtg	38	67.5
DT608217_641_675	cathepsin B	DT608217	ccgtatgccattcctgcttgtgatcatcatgttcc	35	70.9
DT608217_399_433	cathepsin B	DT608217	ttcgagatcaaggagagtgtggcagttgttgggca	35	73.9
CN631613_217_251	acidic glucanase	CN631613	cgctggacatcgtaatgattgccaaccactttgca	35	72.6
CN631613_114_148	acidic glucanase	CN631613	aattcccgttcgaatttaaaagcgttgcagctcct	35	71.1
CN631613_313_347	acidic glucanase	CN631613	tgcgaacactttcaagcccaacggtgctgtctatt	35	73.4
CN631613_2_40	acidic glucanase	CN631613	acgaacaccattgctgattctatctatatatttgaagct	39	67.2
CN631613_241_275	acidic glucanase	CN631613	accactttgcaaatctttgaccggttgtaaaggat	35	69.4
CN629960_357_391	Ribophorin	CN629960	ttatacattcctcgtgaagggttagtgctaccaac	35	68.1
CN629960_53_87	Ribophorin	CN629960	ctgaccagtaagtccatgaactagacgtacaaaag	35	67.0
CN629960_145_181	Ribophorin	CN629960	caagagtttgatgataatctgcctccaatactgaaga	37	67.2
CN629960_81_115	Ribophorin	CN629960	acaaaagcttgatgcacaaccactgactgaccaga	35	71.8
CN629960_429_466	Ribophorin	CN629960	gcaacctcacactcaacaattatgtaatcatctttctc	38	67.1
DT620213_321_355	spondin1	DT620213	tgcaatccatggtcagactatagtgaatgtagcaa	35	68.0
DT620213_441_475	spondin1	DT620213	tcacgcttatgcaatactaaaccatgtcctactga	35	68.0
DT620213_525_560	spondin1	DT620213	gggtatcaatatcgtaatcgtgttggtagttgggaa	36	68.3
DT620213_189_223	spondin1	DT620213	acagcatggagtgattgcagcacctcatgtggttt	35	73.5
DT620213_387_424	spondin1	DT620213	aggaactgctcaattacagacaatattcaagttgatgt	38	67.5
CO539736_161_195	Vitellogenin II precursor	CO539736	gtcatgcagcgtggctctacctcgattcttagtgg	35	72.7
CO539736_2_36	Vitellogenin II precursor	CO539736	cactttacctaaaggaactcccgaaatctcactgg	35	68.6
CO539736_252_287	Vitellogenin II precursor	CO539736	ataaaagtggttccaactcaagactctaagattgca	36	67.1
CO539736_61_95	Vitellogenin II precursor	CO539736	agcccaaatgttatcacggacacgattatttccaa	35	69.0
CO539736_91_127	Vitellogenin II precursor	CO539736	tccaaaccttcgacgatgtggaatatcattttaaact	37	67.6
CV181079_57_91	Apoptosis Regulator BCL-2	CV181079	acttcctaaccgtgtaaacttgaatgcccacaacg	35	70.7
CV181079_154_190	Apoptosis Regulator BCL-2	CV181079	agagtattagctatttatacattggctggttgcatgt	37	67.1
CV181079_244_280	Apoptosis Regulator BCL-2	CV181079	tgggttaaagagtttgtctccatacatttagctgatt	37	67.2
CV181079_322_356	Apoptosis Regulator BCL-2	CV181079	ttgtcgaatgactccaataatcactcatcatggag	35	67.1
CV181079_129_163	Apoptosis Regulator BCL-2	CV181079	tgctgatggcactaaaaattggggaagagtattag	35	67.5
CN774282_47_82	BCL-2 Homologous antagonist/killer 2	CN774282	agaagatgttgcttatgagacttttgctaacattgc	36	67.0
CN774282_359_395	BCL-2 Homologous antagonist/killer 2	CN774282	cattgctacagtttatatgatacaccgtatcacacgt	37	67.2
CN774282_3_39	BCL-2 Homologous antagonist/killer 2	CN774282	agacaagtgttttcagatatgttatgtcgcttgaaca	37	67.6
CN774282_153_187	BCL-2 Homologous antagonist/killer 2	CN774282	gcatttgcaattattaagcgaaatgcgcgtgggtt	35	71.1
CN774282_97_131	BCL-2 Homologous antagonist/killer 2	CN774282	aaaatggtattaattgggggcgtattgtagctctt	35	67.4
CV564390_137_171	Metallothionein	CV564390	ttctattggttcacaatgcgttccaaactactgta	35	67.3
CV564390_233_267	Metallothionein	CV564390	aaattatcgttgtcctgttggataccaatgcgtcc	35	69.3
CV564390_57_91	Metallothionein	CV564390	catatgtcgcagggattaccaccgataacgccgtg	35	73.1
CV564390_183_217	Metallothionein	CV564390	gctgtgtgcaaaccttgcgtaataagttactgcaa	35	70.1
CV564390_390_424	Metallothionein	CV564390	acgtattcgtgttccaagccaattattccaatcta	35	67.2
Ac_1_241_275	Cystatin	BC020532	gctggatcaacccttgaactatcagcattgaagac	35	69.3
Ac_1_349_383	Cystatin	BC020532	atctgtcgccacatacgtaaagggacaccaatttt	35	70.2
Ac_1_167_206	Cystatin	BC020532	caaattactttattaaggtcaatgttggtgatgggaactt	40	67.2
Ac_1_393_427	Cystatin	BC020532	agggtttggaaatcttgctatgaacatgcaacctc	35	69.5
Ac_1_13_47	Cystatin	BC020532	atggcgatgtgcggtggaactggccaactaaaaca	35	74.9
Ac_2_161_195	L-arginine:glycine amidinotransferase	X86401	gatgacctttatgatcaggagtatttgtcgctacc	35	67.1
Ac_2_209_243	L-arginine:glycine amidinotransferase	X86401	cgtaagttagcaatgaccgaaggcaagactatctt	35	68.5
Ac_2_393_427	L-arginine:glycine amidinotransferase	X86401	cttttgatgatccaaatccacttcacattgatgca	35	67.1
Ac_2_329_363	L-arginine:glycine amidinotransferase	X86401	gactttgggattgaatggatgcgccgtcatcttgg	35	72.6
Ac_2_254_288	L-arginine:glycine amidinotransferase	X86401	gaaccatgttttgatgccgctgacttcatgagagc	35	71.4
Ac_3_25_59	Agmatinase	AY057097	acctgttggtctagttcatgtcgatgctcattcac	35	70.0
Ac_3_285_319	Agmatinase	AY057097	agcagatgggcaatggacccgtttatatatccttt	35	69.7
Ac_3_97_131	Agmatinase	AY057097	tggaacaccatttcgtcgagcagttgaggaaggcc	35	74.5
Ac_3_209_243	Agmatinase	AY057097	aagcaggctttcaggttagttatggcacatgagtg	35	70.4

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
Ac_3_325_359	Agmatinase	AY057097	tgatggcttacatccagcattcgctccaggaacag	35	73.0
Ac_4_145_179	Dolichyl-phosphate mannosyltransferase polypeptid	BC032223	tgggtgttattcgttagcaaccataggataccggg	35	70.9
Ac_4_313_347	Dolichyl-phosphate mannosyltransferase polypeptid	BC032223	cagacataagtgtggtcaccgtgaaagattgccag	35	70.7
Ac_4_254_289	Dolichyl-phosphate mannosyltransferase polypeptid	BC032223	agtttcactctcaacctatttgaatagtttcaggcc	36	67.5
Ac_4_390_424	Dolichyl-phosphate mannosyltransferase polypeptid	BC032223	ctagtctattgatggagttaataggtcccttcggc	35	67.8
Ac_4_9_44	Dolichyl-phosphate mannosyltransferase polypeptid	BC032223	tgacagagtggattgtacttgctgggcttgccttttt	36	73.1
Ac_5_73_107	Retinol dehydrogenase 8 (all trans)	AK024022	actggaagacgagggagcgaaatatctgggcaaca	35	73.6
Ac_5_118_154	Retinol dehydrogenase 8 (all trans)	AK024022	caaacagatggatgttagcaatgatgaatcagtgaag	37	67.2
Ac_5_17_51	Retinol dehydrogenase 8 (all trans)	AK024022	gcagagaaacgcttcaaagtttatgccaccatgag	35	70.1
Ac_5_43_77	Retinol dehydrogenase 8 (all trans)	AK024022	caccatgagaaatctgtcgaagaaaggacaactgg	35	69.2
Ac_5_97_131	Retinol dehydrogenase 8 (all trans)	AK024022	tctgggcaacactttgattgtcaaacagatggatg	35	69.6
Ac_6_161_195	Alpha X integrin	TBS	accaattccaacggcgattctatgcacacctttat	35	70.5
Ac_6_17_51	Alpha X integrin	TBS	caggcggtatatcctccatcgatgctacaagtgat	35	70.6
Ac_6_209_243	Alpha X integrin	TBS	gataacttggctgtacggctttgacttcgggttgg	35	72.1
Ac_6_305_339	Alpha X integrin	TBS	gttgaatacatccttgccgactgtctccaatgcaa	35	70.7
Ac_6_92_126	Alpha X integrin	TBS	acttttggcattgacaacgcgattagggtctccag	35	72.0
Ac_7_81_115	Subtilisin/kexin isozyme	AL133583	actcatccgggactagcatagcctcgtttccatct	35	72.9
Ac_7_141_175	Subtilisin/kexin isozyme	AL133583	tcaaccatcagggaaacgaggtcgtgacgggagag	35	74.8
Ac_7_9_43	Subtilisin/kexin isozyme	AL133583	ctccttggacaactgctggccatgaaagtgcaatt	35	72.7
Ac_7_173_207	Subtilisin/kexin isozyme	AL133583	gagactaagaccgaggaagacgtggcagtcttggg	35	73.5
Ac_7_191_225	Subtilisin/kexin isozyme	AL133583	gacgtggcagtcttggggctttatcaggttccagg	35	74.5
Ac_8_304_338	Zinc finger protein 294	AB018257	aacacaagtcttgctcttatgctggcgtggaaact	35	71.9
Ac_8_196_231	Zinc finger protein 294	AB018257	ttactagagatgatcgatttgacttgttcacgcatg	36	67.4
Ac_8_27_61	Zinc finger protein 294	AB018257	tgacctcatggcgcactgcctacctttgctaaagt	35	74.2
Ac_8_69_103	Zinc finger protein 294	AB018257	gcatgctcttcagtttgcggcatatcatttgctgt	35	71.7
Ac_8_247_281	Zinc finger protein 294	AB018257	gaagctcaagtgccgtttggcgagcatctggagat	35	74.7
Ac_9_161_195	N-methylpurine DNA glycosylase	AF499437	cgactcgacgcatatcagcaaagtaccgttgaaga	35	71.3
Ac_9_345_379	N-methylpurine DNA glycosylase	AF499437	gaatctggcgcatgcgttttgatccgatcagtgga	35	73.6
Ac_9_249_283	N-methylpurine DNA glycosylase	AF499437	agaacagaacgcaacgagccgatgttcatggttcc	35	73.4
Ac_9_209_243	N-methylpurine DNA glycosylase	AF499437	caagaacactaaggcagagcttaccgcgtttctag	35	70.3
Ac_9_5_39	N-methylpurine DNA glycosylase	AF499437	gagaccaacagctcgcagaacacaacgatcgtttc	35	72.3
Ac_10_333_367	Glutathione S-transferase	X68677	aagagtgaaaacgttactttgcgatggctgtcctt	35	70.5
Ac_10_158_192	Glutathione S-transferase	X68677	ctccttgcttgaaccccatgacaacctgaaggaat	35	71.8
Ac_10_193_227	Glutathione S-transferase	X68677	ttgcaaaaaggattgaggaactacctgccattgat	35	69.2
Ac_10_137_171	Glutathione S-transferase	X68677	tcagcacaagatttttgatgcctccttgcttgaac	35	70.0
Ac_10_211_245	Glutathione S-transferase	X68677	aactacctgccattgatgcatacagaaaatctgat	35	67.3
Ac_11_305_339	Glutathione S-transferase (michrosomal)	BC005964	tgctgggtgcaatctaatcttagacaagcaagtga	35	69.9
Ac_11_91_125	Glutathione S-transferase (michrosomal)	BC005964	gtggagaactatcccatattcttgtacctgctcct	35	68.9
Ac_11_17_51	Glutathione S-transferase (michrosomal)	BC005964	gcatagagtacccaaggatgtatgtcgacaaggag	35	69.4
Ac_11_233_267	Glutathione S-transferase (michrosomal)	BC005964	gcatgcgaggaggctttggctacttaggactgctg	35	74.6
Ac_11_135_169	Glutathione S-transferase (michrosomal)	BC005964	cttggaacaccctattacaagctccgtagctggaa	35	71.0
Ac_12_53_87	Methyl-CpG binding protein 2	BC032638	aatcctctcgagatggacgaaaagcctatacgaat	35	69.0
Ac_12_5_39	Methyl-CpG binding protein 2	BC032638	gagaataaggccaacgtgcttgaggacttgaagtg	35	70.7
Ac_12_253_287	Methyl-CpG binding protein 2	BC032638	tgattttagaagtgggacatattgcagacggagca	35	69.8
Ac_12_125_159	Methyl-CpG binding protein 2	BC032638	atcgtgatgagaaagtctggactgtcagctggaag	35	70.9
Ac_12_305_340	Methyl-CpG binding protein 2	BC032638	aaggagcagagttatgggcgagacttaaatggagga	36	71.7
Ac_13_121_155	Cysteine rich FGF receptor	U28811	cgcagaggatatggtaactgtatggttagagcagt	35	69.1
Ac_13_73_107	Cysteine rich FGF receptor	U28811	agacagtgtgcaaacagtatcgctgtggatgattc	35	70.4
Ac_13_289_324	Cysteine rich FGF receptor	U28811	cttgggtgataaaaccattaaagcaagtgttcagag	36	67.2
Ac_13_385_419	Cysteine rich FGF receptor	U28811	gaggcaaactagactcaggtggtgcaccagttctt	35	72.7
Ac_13_194_229	Cysteine rich FGF receptor	U28811	ttttacctggcaaatgatgttcttcaaaatagtcgc	36	67.3
Ac_14_65_99	Acid phosphatase type 5	J04430	tcgtcattggatgcgctgcagttatggatacaagc	35	72.1
Ac_14_217_251	Acid phosphatase type 5	J04430	ctagatgcgcatggatcgatgttgtattcacgaaa	35	69.0
Ac_14_177_211	Acid phosphatase type 5	J04430	agtggtacgcgctggagagtccattttgaccttag	35	72.5
Ac_14_45_79	Acid phosphatase type 5	J04430	aaattccacagttgagtatttcgtcattggatgcg	35	68.3
Ac_14_105_139	Acid phosphatase type 5	J04430	gcatttgaatgccgtacctaaaggaagtttgcatt	35	68.9
Ac_15_129_163	Serine dehydratase-related	BC009849	caacttgactgcatggattcatgttacctgaagaa	35	67.8
Ac_15_209_243	Serine dehydratase-related	BC009849	ttccctacacctcgaatcttgaatgacgacactgg	35	70.9
Ac_15_77_113	Serine dehydratase-related	BC009849	tctgtcaaggtgattaacagcttacattattgactgt	37	67.1
Ac_15_169_203	Serine dehydratase-related	BC009849	catgtgattgcatcccttacgcatggccttttggc	35	73.3
Ac_15_37_71	Serine dehydratase-related	BC009849	aaaggaggatgggccaatgctcaagacttccgtta	35	72.6
Ac_16_77_111	Inositol polyphosphate 4-phosphatase	U26398	gcctttgttccgaccaatctacacgttcagcggat	35	73.2
Ac_16_9_43	Inositol polyphosphate 4-phosphatase	U26398	tcgtgccgcttattcctttacaagatacgtgcttc	35	70.4
Ac_16_129_163	Inositol polyphosphate 4-phosphatase	U26398	gcgaaggaggaacattgtatgacgtagtgactgtt	35	69.9
Ac_16_101_135	Inositol polyphosphate 4-phosphatase	U26398	gttcagcggatgaacgttatgcaggacggcgaagg	35	74.9
Ac_16_57_91	Inositol polyphosphate 4-phosphatase	U26398	tcagaaatgacaagaaattggcctttgttccgacc	35	69.7
Ac_17_33_67	Kinectin 1	Z22551	caggaagcaacgtcaagggtgtctgagctggaaga	35	74.0
Ac_17_77_111	Kinectin 1	Z22551	aggatttgaatatcagttgaaggtgtcacaggaaa	35	67.0

Table A.1 (continued).

Probe	Gene Name	Accession	Sequence	bp	Tm
Ac_17_245_279	Kinectin 1	Z22551	atctgcgaatgtccaggaggatttattaaaggtga	35	68.1
Ac_17_143_177	Kinectin 1	Z22551	tcttcaagaggaacttagattggcacaggaacaag	35	68.6
Ac_17_405_439	Kinectin 1	Z22551	gctatggaagccttgtctgaagctgagaaaactgc	35	70.9
Ac_18_266_302	Thymosin beta-10	M92383	ttgcctatacctgaaacgaattaagtgttagaaaggg	37	67.4
Ac_18_309_343	Thymosin beta-10	M92383	tccagtttcgaagtagggtattgtgatgccttcat	35	69.5
Ac_18_111_145	Thymosin beta-10	M92383	atttgacaaatcgaagttgaagcatgccgagacta	35	69.0
Ac_18_364_398	Thymosin beta-10	M92383	tccggcgatagttcaatcagaagttatcaaattgt	35	67.2
Ac_18_409_443	Thymosin beta-10	M92383	tgctgtcttccattgtaagcactctcgtttaggtc	35	69.9
Ac_19_97_131	Profilin	S13198	ctatcaacgcgactacatgcacttgtactagccta	35	69.3
Ac_19_337_371	Profilin	S13198	tctgcaattacagctactcctttgttcacattgcc	35	69.4
Ac_19_241_276	Profilin	S13198	ggtgaacaatttagtaacaaagtgagagtcacacct	36	67.5
Ac_19_54_88	Profilin	S13198	tatttcagactgaaggtaaactcgaaatcacaccg	35	67.2
Ac_19_361_395	Profilin	S13198	ttcacattgccttgctgacttccctcacgggtatg	35	73.1
Ac_20_201_235	mu-protocadherin	AAF70456	atacaacttggtcgccagtacctggtattcgaggg	35	72.1
Ac_20_257_291	mu-protocadherin	AAF70456	tcaacatcaggctcaaacaagttgctagttgggtt	35	70.5
Ac_20_325_359	mu-protocadherin	AAF70456	agcagccattgcgggatttgtgtggttcaggagaa	35	74.7
Ac_20_8_42	mu-protocadherin	AAF70456	cctaagcgagaaggcagtgcactagggaaatttcc	35	71.8
Ac_20_365_400	mu-protocadherin	AAF70456	aaccgtcagtttagttaccagaaaccagttctttac	36	67.4
Ac_21_169_203	RING-H2 finger protein RHA1a	AP000616	caccggtggttttgcaaattttataaccgcactca	35	70.3
Ac_21_141_178	RING-H2 finger protein RHA1a	AP000616	gtaaaatgttcagtaagatctttagtttcaccggtggt	38	67.7
Ac_21_194_229	RING-H2 finger protein RHA1a	AP000616	accgcactcattataaccttcatttttaagctgagt	36	67.5
Ac_21_223_262	RING-H2 finger protein RHA1a	AP000616	gctgagttaaaaagctaatttttattagcaaggcaaaacg	40	67.5
Ac_21_275_313	RING-H2 finger protein RHA1a	AP000616	tgccacaaaaacaacaattaaagattgctaatactctgg	39	67.7
Ac_22_179_213	glutaredoxin	P55143	ttgaaagactatgtgttgttcgaactcgacacgat	35	68.5
Ac_22_244_278	glutaredoxin	P55143	gaaagagattacaggcgcaagatccgttccaaggg	35	71.9
Ac_22_9_43	glutaredoxin	P55143	cgcatcatccgttaatttgcaatagccgaagaagc	35	70.3
Ac_22_152_186	glutaredoxin	P55143	gcgaaatcagcgttgggcgagactggcttgaaaga	35	75.0
Ac_22_209_243	glutaredoxin	P55143	acgatggatgatggcgatgcgtatcaagatgcctt	35	72.8
Ac_23_177_211	SH3 Domain-binding glutamic acid-rich protein	Q9NFP5	cagattgtaaatggtagcacgtactgtgggacatt	35	68.8
Ac_23_293_327	SH3 Domain-binding glutamic acid-rich protein	Q9NFP5	ctgataaacaccagcctacaacagagtctttgaca	35	68.5
Ac_23_101_135	SH3 Domain-binding glutamic acid-rich protein	Q9NFP5	agttgatgtggcacaagataatgaagctctaggga	35	69.3
Ac_23_217_251	SH3 Domain-binding glutamic acid-rich protein	Q9NFP5	aatttgaaactgccattgagcaagagactcttcat	35	67.9
Ac_23_145_179	SH3 Domain-binding glutamic acid-rich protein	Q9NFP5	agcttgttgatgacgaagaggcattagctcctcag	35	71.3
Ac_24_145_179	PDGF associated protein	XP_004846	tgattggtgctaagtgtcgaatgacccatctcgta	35	70.6
Ac_24_265_299	PDGF associated protein	XP_004846	agatcgcagctatctccaatacgaagccggtcaac	35	72.5
Ac_24_21_55	PDGF associated protein	XP_004846	ggctcggagcgatttggcaagactagcgcttatac	35	73.2
Ac_24_337_371	PDGF associated protein	XP_004846	gcttggttattggtgatagaaggtatcagtgaagc	35	67.6
Ac_24_185_219	PDGF associated protein	XP_004846	tgaacagcgaggcaagaatgtacaccagatgaact	35	71.2
Ac_25_10_44	calmodulin	P02594	gttctttcggctgttgggaaggttgagtcggaatc	35	72.1
Ac_25_193_227	calmodulin	P02594	tcacgactgaagagttattgctcgtcatgaagaac	35	68.5
Ac_25_369_403	calmodulin	P02594	gaggcattcaaggcttacgacacggacaacaaggg	35	73.5
Ac_25_307_341	calmodulin	P02594	ttgaggagtttgcggagctcatggtcaaacagatg	35	71.9
Ac_25_66_105	calmodulin	P02594	cttgtacagattaagtgaaagcgtctttgaatatcaaaca	40	67.1
Ac_26_129_163	spectrin alpha II chain	S61217	ggaacacgttgactcatattcgataagccagcggt	35	71.5
Ac_26_89_123	spectrin alpha II chain	S61217	tcaaggctttgcaacaggacgctgaccaactgata	35	73.0
Ac_26_65_99	spectrin alpha II chain	S61217	ccctcacggcttatgaagagaagatcaaggctttg	35	70.4
Ac_26_157_194	spectrin alpha II chain	S61217	cagcggtgcgaaggagttgtggaaaactggaaaagact	38	74.6
Ac_26_109_143	spectrin alpha II chain	S61217	gctgaccaactgatacagcaggaacacgttgactc	35	71.9
Ac_28_305_339	Hsp 70	AAF75876	aaaggaattgtttaccgaaagcgtattgcccgacg	35	71.2
Ac_28_13_48	Hsp 70	AAF75876	ccagaagtgttggcagcgtttcaggatccagaggtg	36	74.6
Ac_28_351_385	Hsp 70	AAF75876	aatttgacactgacttcatcagacaacttccagga	35	68.4
Ac_28_65_99	Hsp 70	AAF75876	atgtcagccagaacccggcgaatatgtccaaatac	35	71.7
Ac_28_389_423	Hsp 70	AAF75876	tctctctctagacttttcaatagctcccaaatgcg	35	68.1
Ac_29_413_447	Ran-GTP binding protein	AAC14260	aagccaacggaaagggcaagttgcgagacttttgc	35	74.5
Ac_29_161_195	Ran-GTP binding protein	AAC14260	agcatgtgttggacaactaactgaagaccatcagg	35	70.2
Ac_29_113_147	Ran-GTP binding protein	AAC14260	agttctttccattactcgacaaatccagagtgctg	35	68.7
Ac_29_301_335	Ran-GTP binding protein	AAC14260	agagaattgttctgattttaggcatgaaatgctgc	35	67.2
Ac_29_329_363	Ran-GTP binding protein	AAC14260	atgctgcggcacattttccgttgacttgctatctt	35	72.4
Ac_30_189_224	Mnn4p - involved in mannose metabolism	NP_012721	ggttacgaaaagtgaacgcagttctgaagaatttga	36	68.3
Ac_30_101_135	Mnn4p - involved in mannose metabolism	NP_012721	aggacagtgaacaaagttctgaagaatctgacagg	35	68.5
Ac_30_286_320	Mnn4p - involved in mannose metabolism	NP_012721	agtgatggaggttacgacggtaaaaatcgaggaga	35	70.2
Ac_30_259_293	Mnn4p - involved in mannose metabolism	NP_012721	cacagttctgaggaatcggagaaagaaagtgatgg	35	68.9
Ac_30_231_267	Mnn4p - involved in mannose metabolism	NP_012721	agtgactagaagagattggaaaaatgaacacagttct	37	67.0
Ac_31_17_51	NADH-ubiquinone oxidoreductase	P34943	tggtaagccgaggtgtcgacaaattgtgcgggttc	35	74.7
Ac_31_341_379	NADH-ubiquinone oxidoreductase	P34943	tgaagaattctaattgtgtcattaatttgattgggcgac	39	67.4
Ac_31_152_190	NADH-ubiquinone oxidoreductase	P34943	cagtgtttggaagtactggattcttaggaagatatgtgc	39	68.9
Ac_31_81_115	NADH-ubiquinone oxidoreductase	P34943	tgagagaaatgtcagttacttgccaaaggctggag	35	70.2
Ac_31_301_335	NADH-ubiquinone oxidoreductase	P34943	gattacaatctcaaagatcctgaatctgtggccaa	35	67.4

Table A.2. Number of genes from each species represented on the array.

Organism	# Genes
Acropora cervicornis	10
Acropora digitifera	2
Acropora donei	1
Acropora formosa	1
Acropora millepora	34
Acropora prolifera	1
Acropora yongei	1
Aiptasia pallida	1
Alcyonium paessleri	1
Anemonia viridis	4
Anthopleura elegantissima	9
Aurelia aurita	1
Dendronephthya klunzingeri	3
Galaxea fascicularis	1
Hydra magnipapillata	9
Hydra vulgaris	1
Hydractinia echinata	2
Montastraea annularis	29
Montastraea cavernosa	2
Montastraea faveolata	20
Pocillopora damicornis	1
Stylophora pistillata	2
Symbiodinium sp.	12

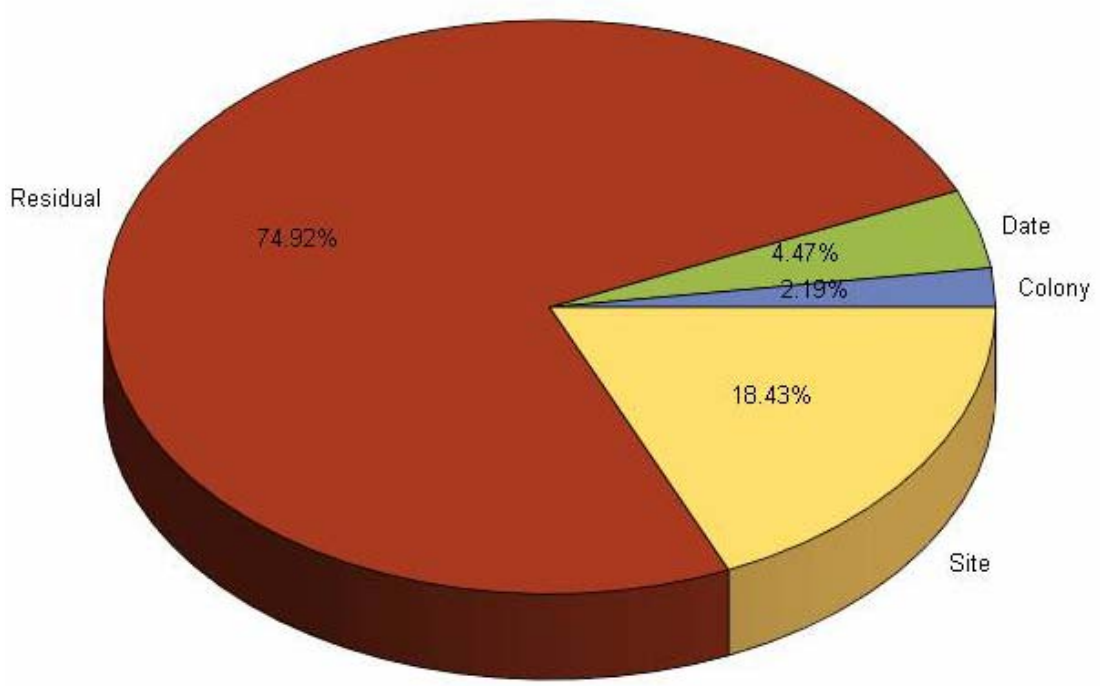


Figure A.1. Pie graph of variance within the data produced by principal component analysis. Sources of variability in principal components 1 through 3 are 81.99% of the total variance. Residual-74.92% (red), Date-4.47% (green), Site-18.43% (yellow), Colony-2.19% (blue).

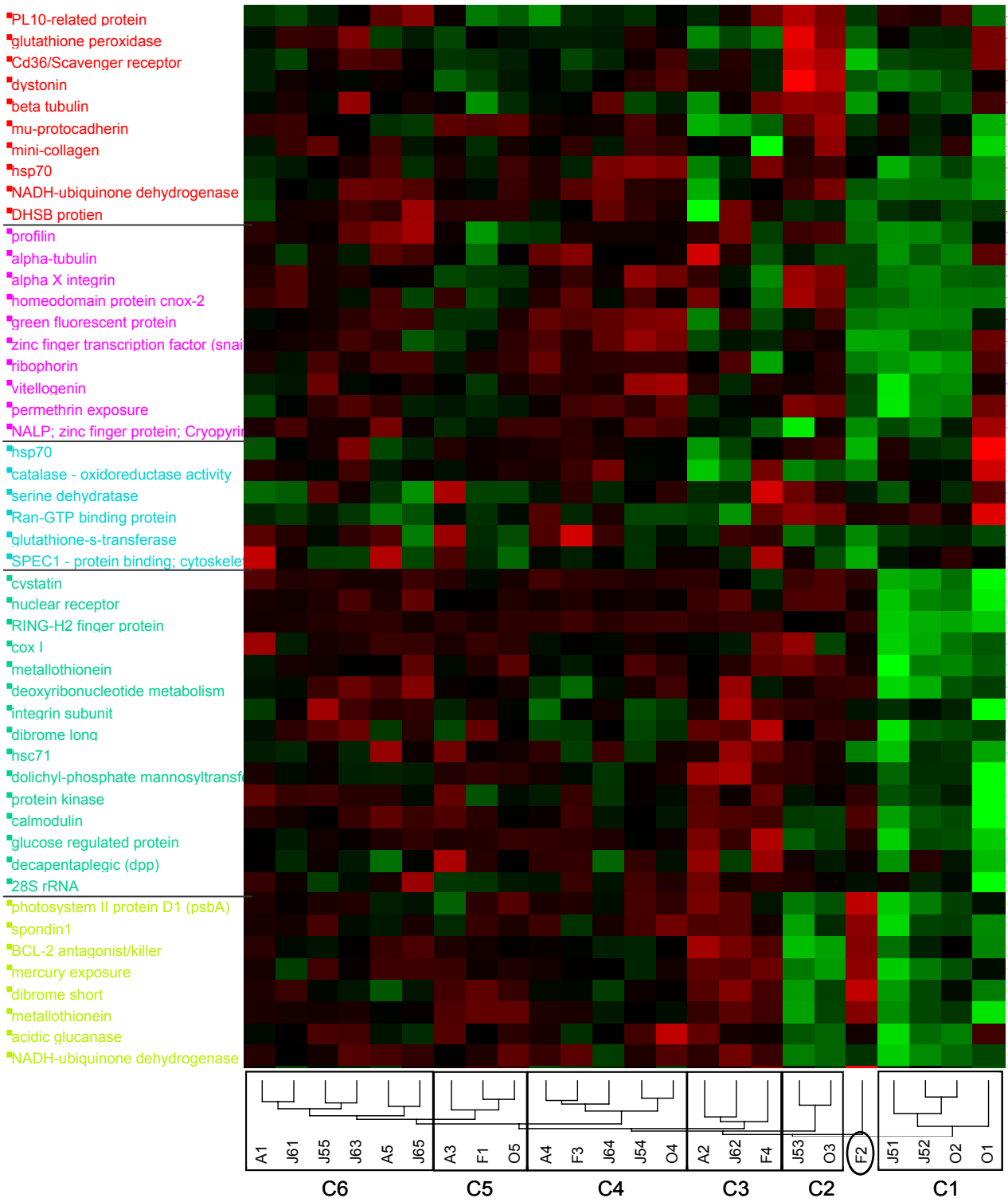


Figure A.2. Heat map of Ward's hierarchical cluster analysis. Gene expression patterns between samples of M. *cavernosa* are clustered based on deviation from the StdLsmean produced by the ANOVA between samples. Ten clusters of genes and six clusters of samples with similar profiles were generated. Red indicates a value > StdLSmean, green indicates a value < StdLSmean, and black is not different from the mean.

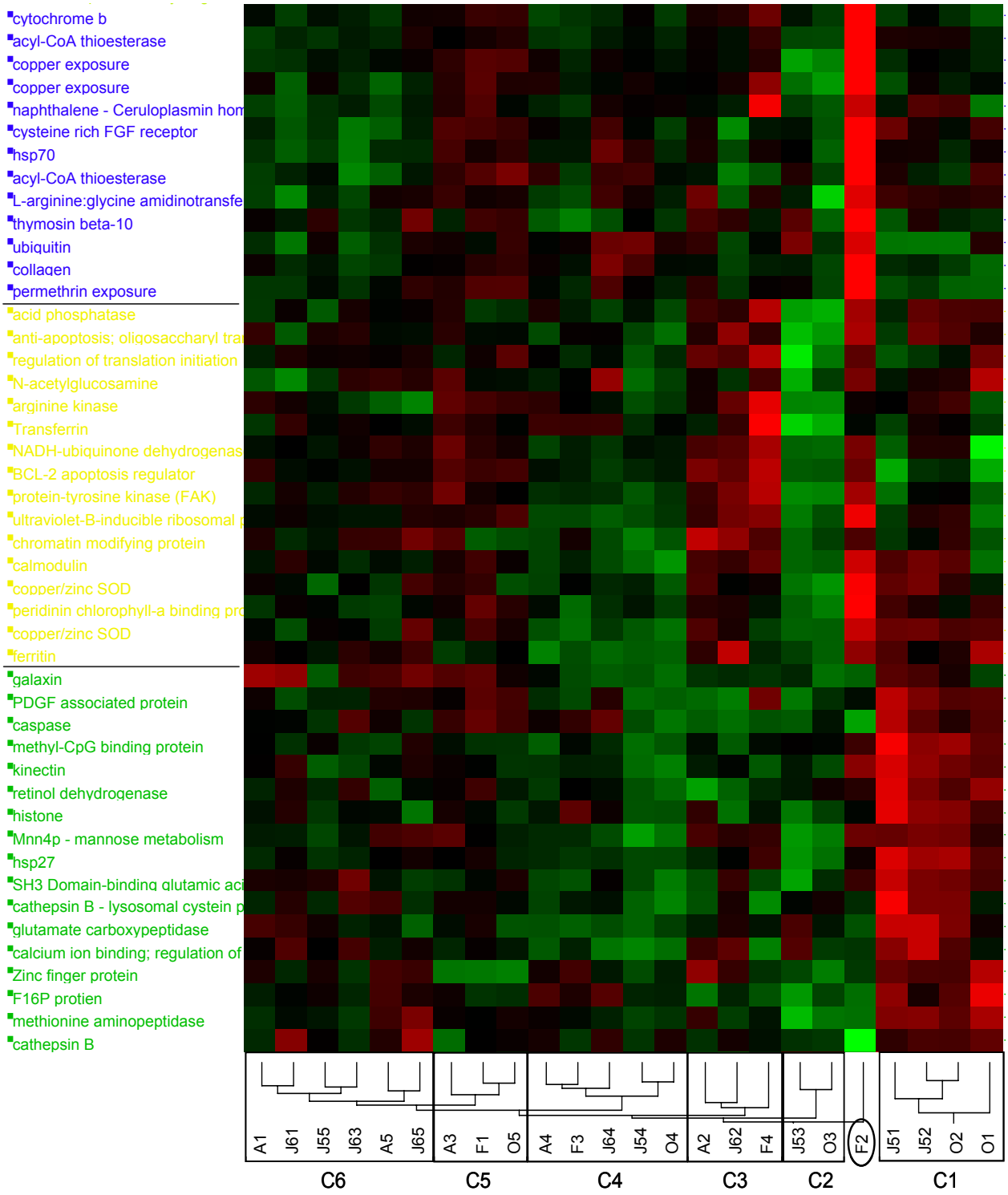


Figure A.2 (continued).

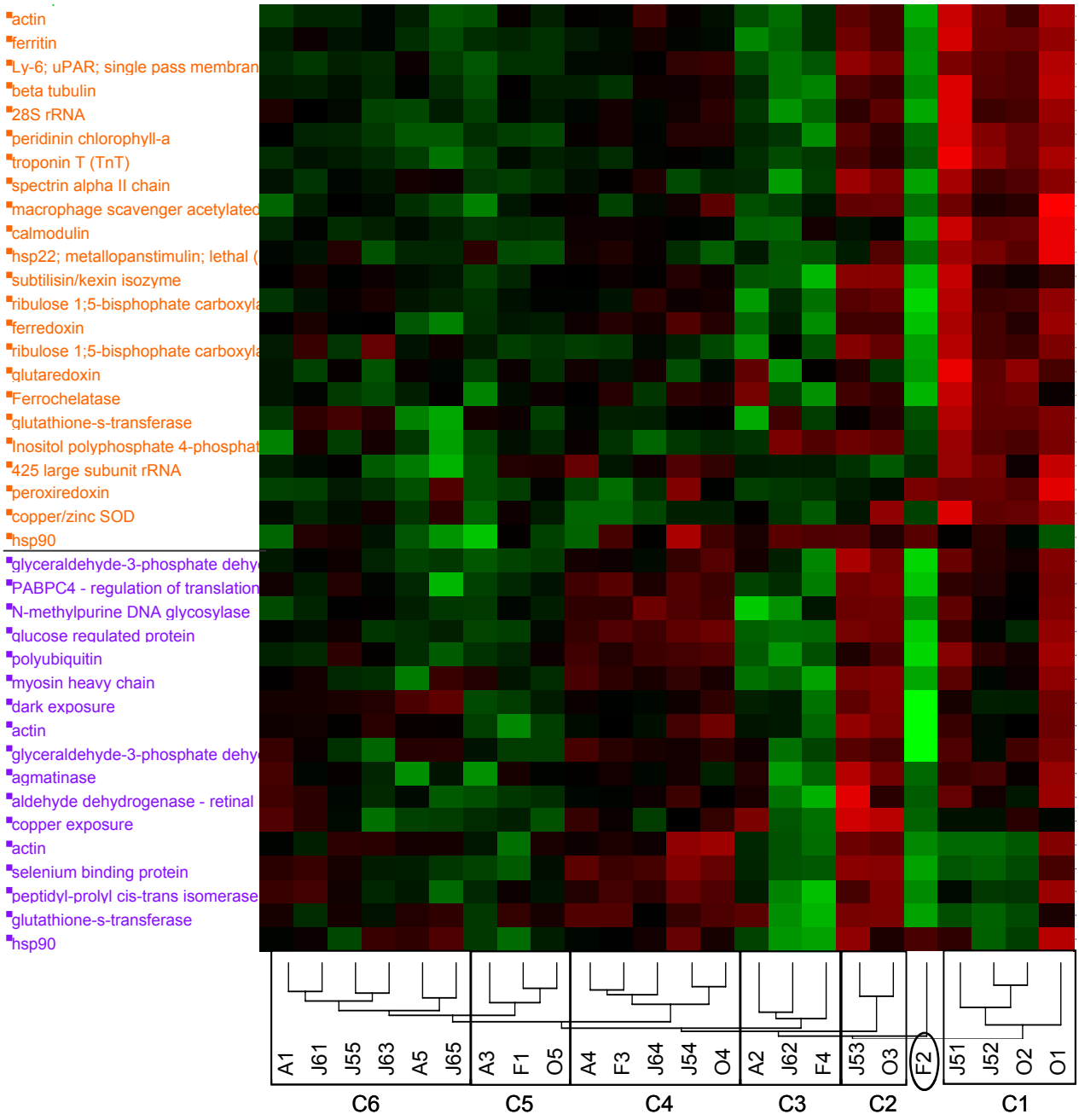


Figure A.2 (continued).

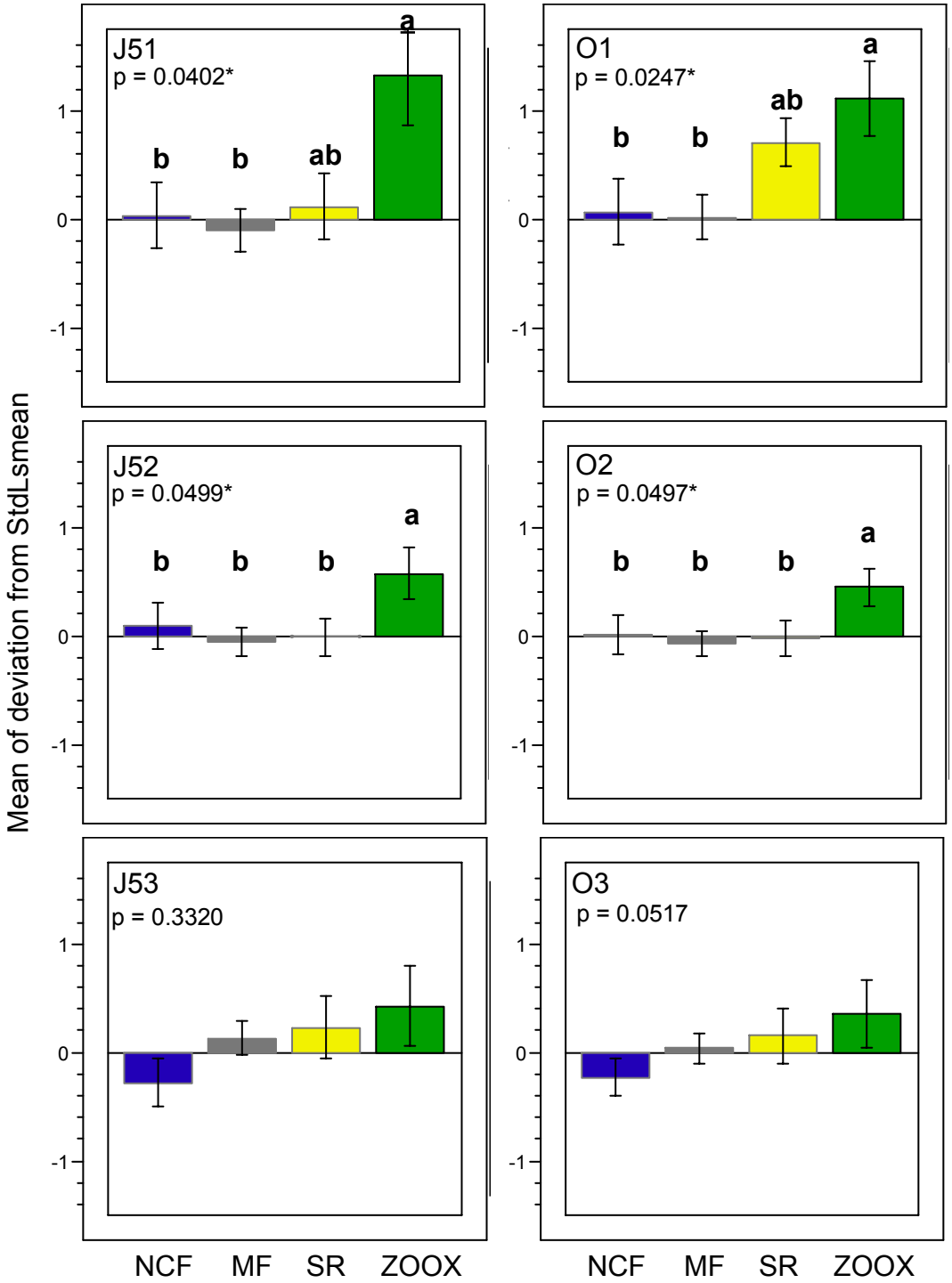


Figure A.3a. Clusters 1 and 2. Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample. Cluster 1 = J51, J52, O1, and O2. Cluster 2 = J53 and O3.

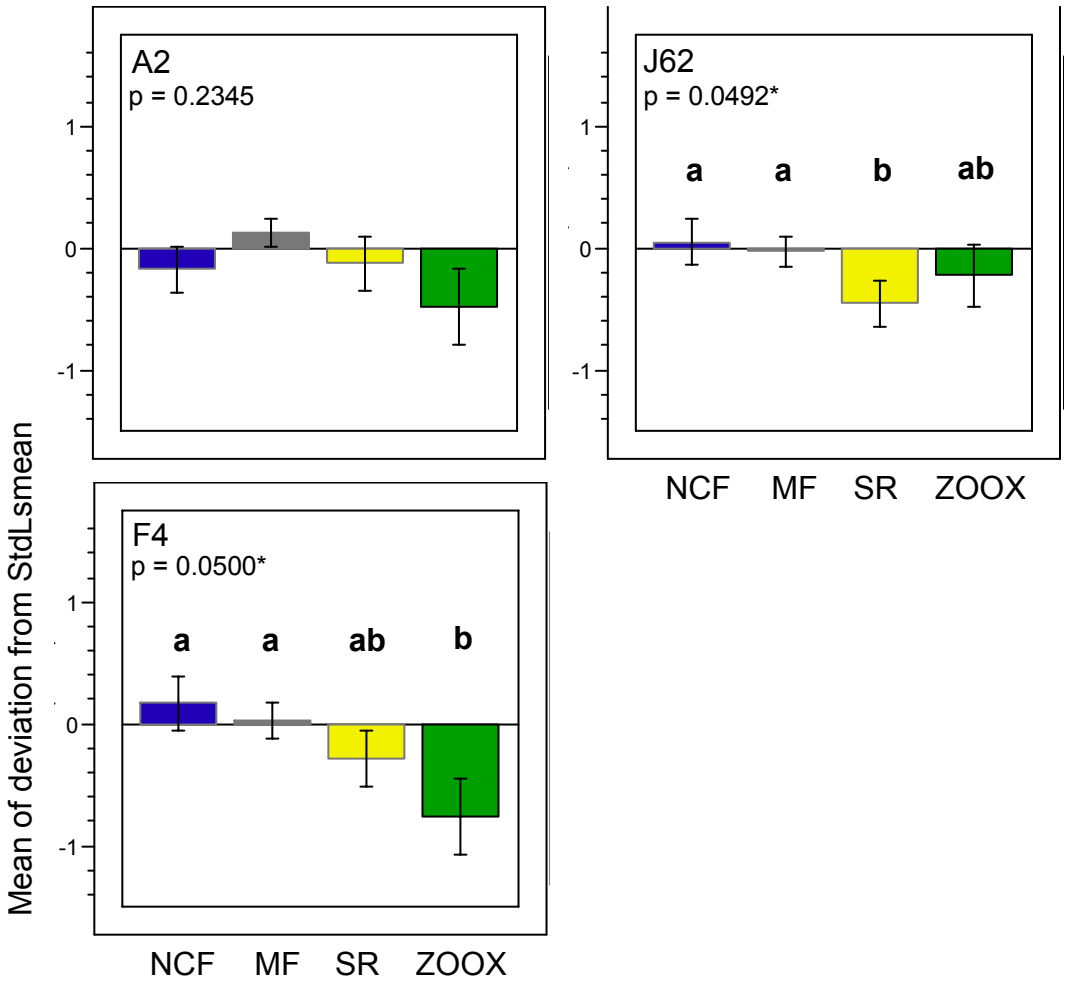


Figure A.3b. Cluster 3. Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample.

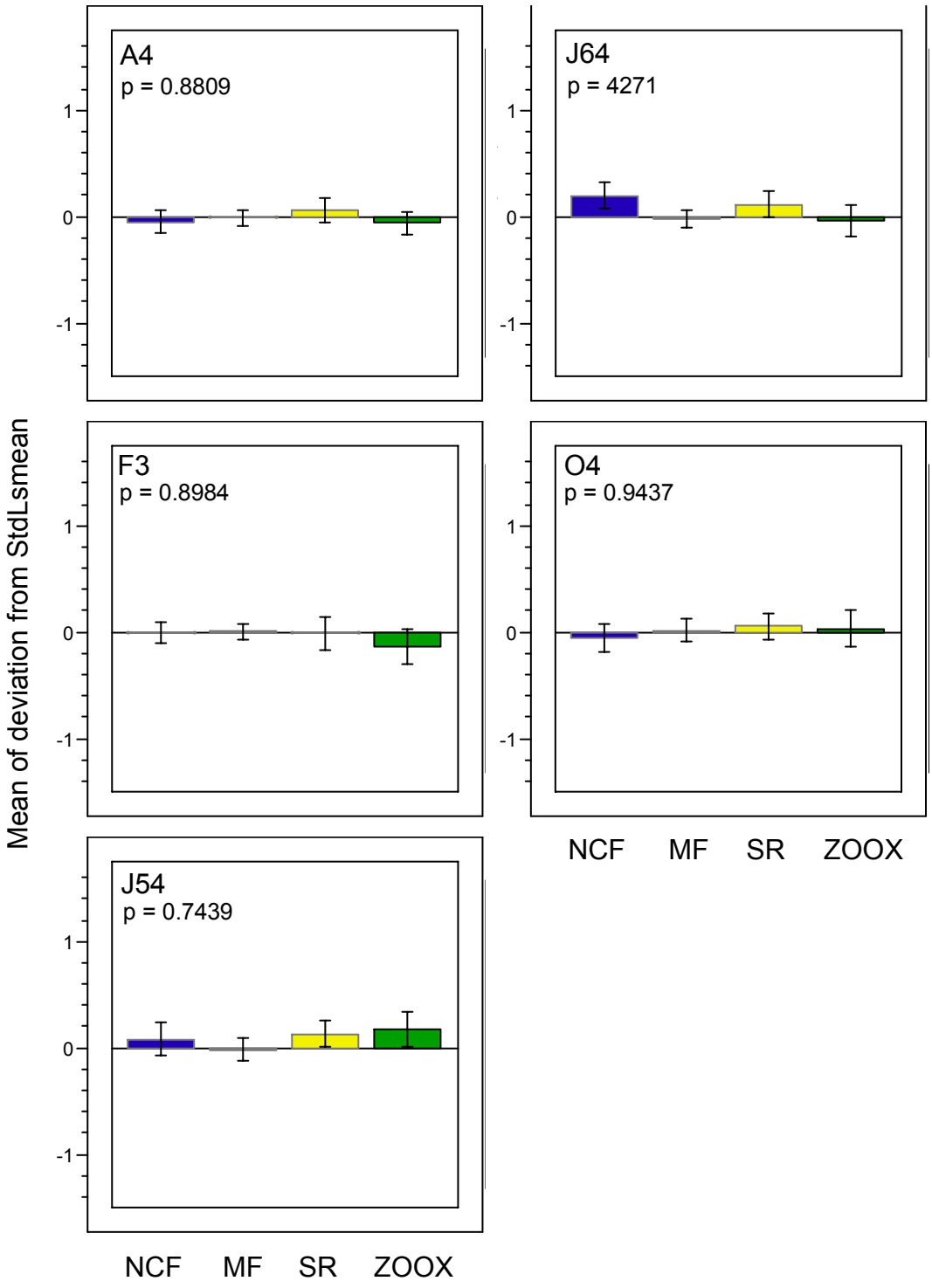


Figure A.3c. Cluster 4. Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample.

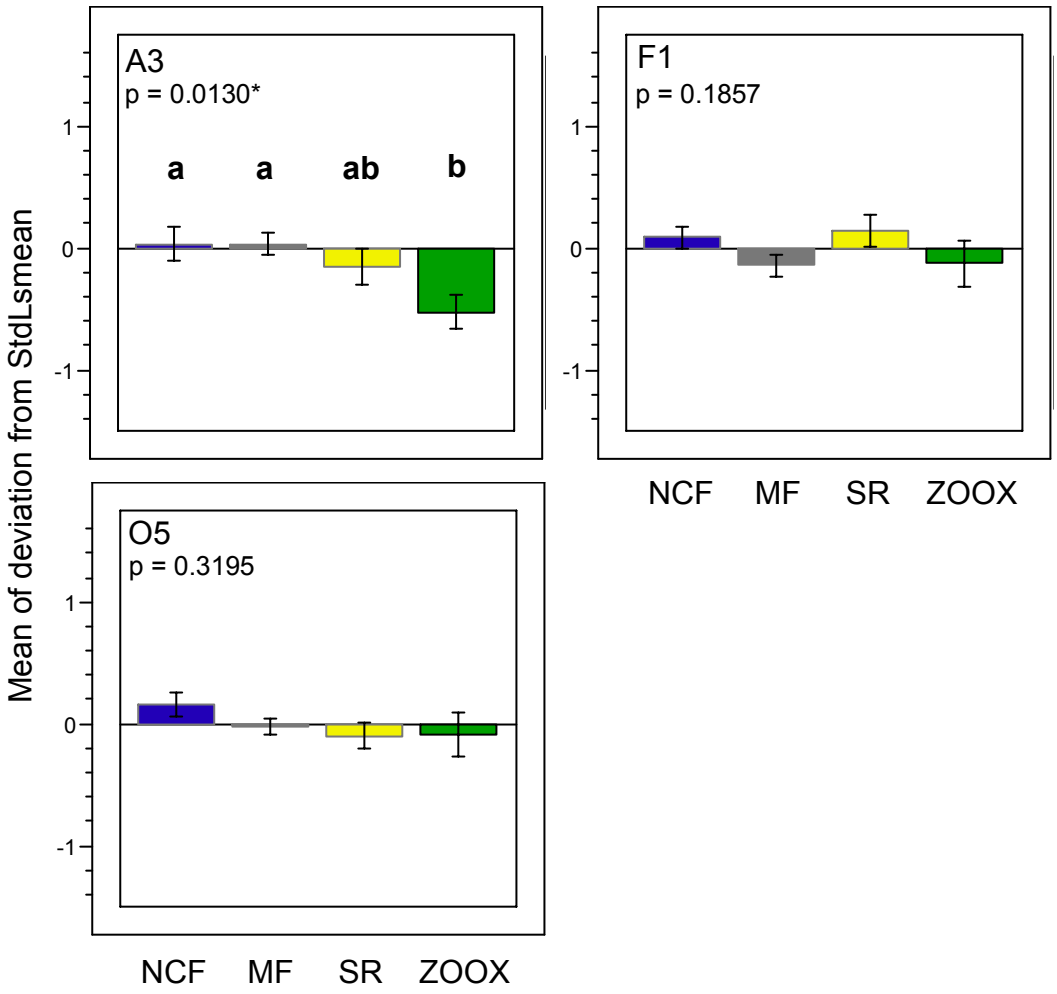


Figure A.3d. Cluster 5. Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample.

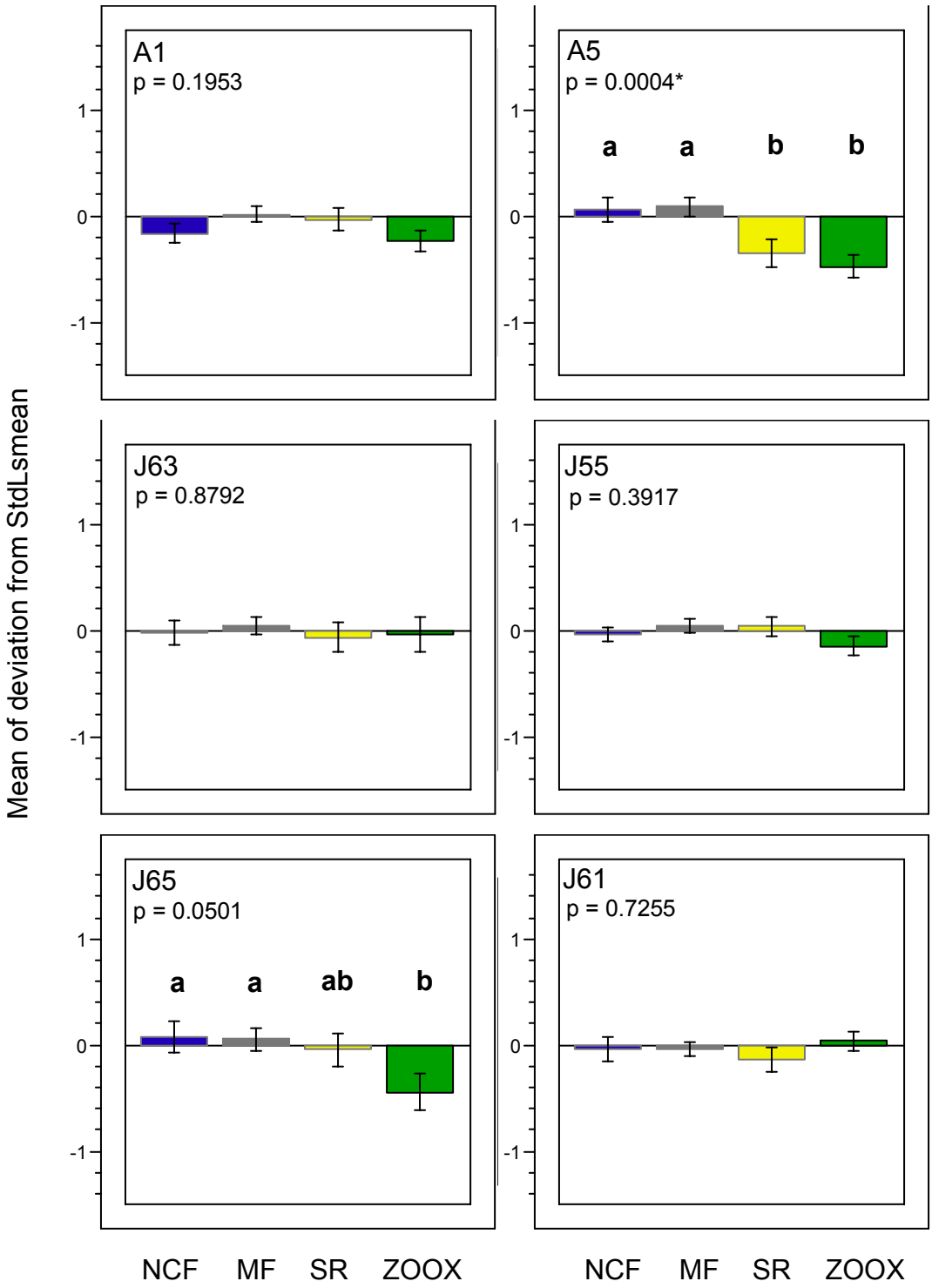


Figure A.3e. Cluster 6. Deviation of significant genes (produced by multivariate ANOVA) from the standard least squares mean (StdLsmean = 0) by group; normal cellular functioning genes (NCF), multifunctional genes (MF), stress response genes (SR), and symbiont specific genes (ZOOX). Significant differences between groups are indicated by p-value (Welch ANOVA). Groups not connected by the same letter are statistically different (Tukey-Kramer). Each graph represents a different sample.

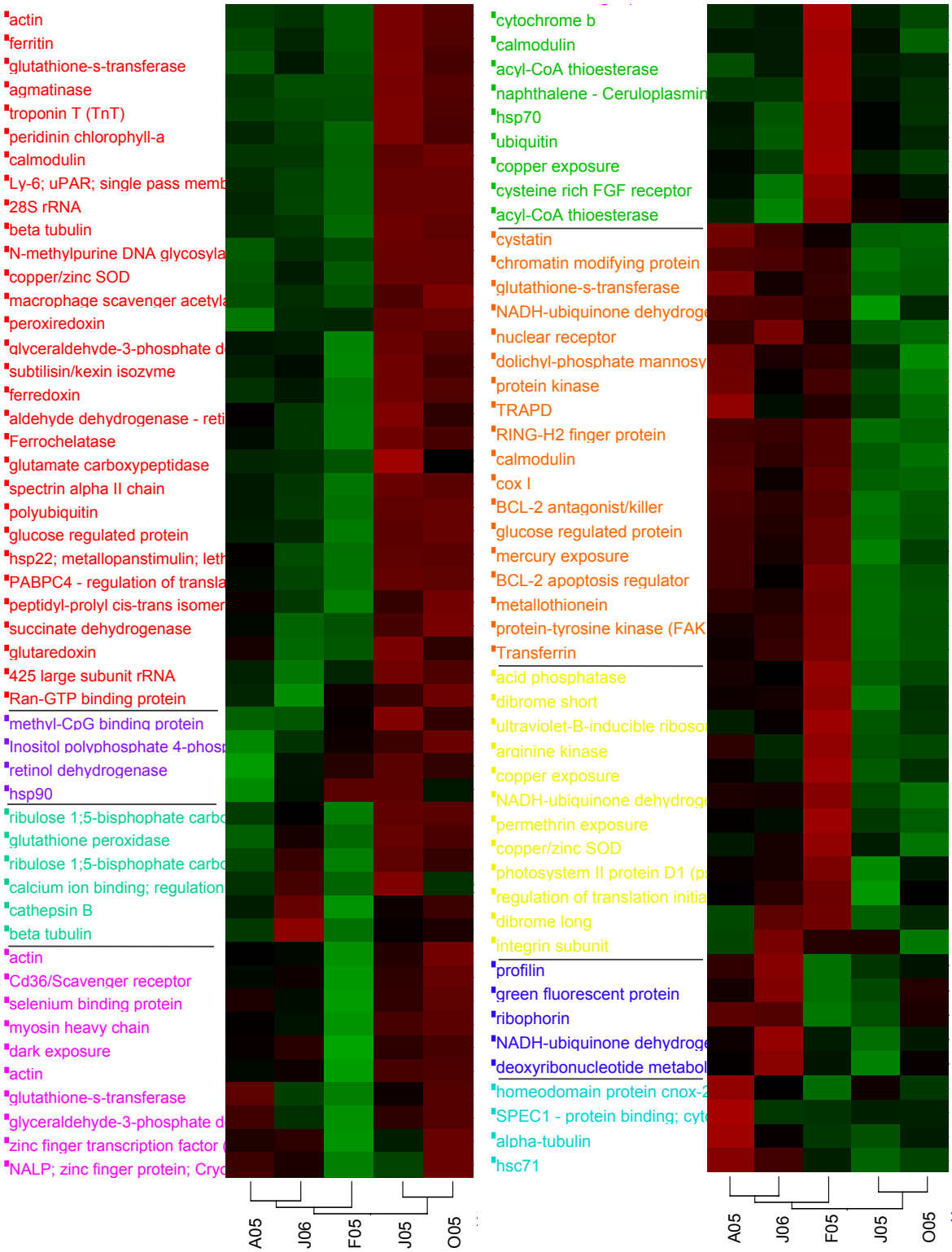


Figure A.4. Heat map of Ward's hierarchical clustering analysis of the deviations from the StdLsmean produced by the ANOVA between dates. Nine clusters of genes and two clusters of dates with similar profiles were generated. Red indicates a value > StdLSmean, green indicates a value < StdLSmean, and black is not different from the mean.

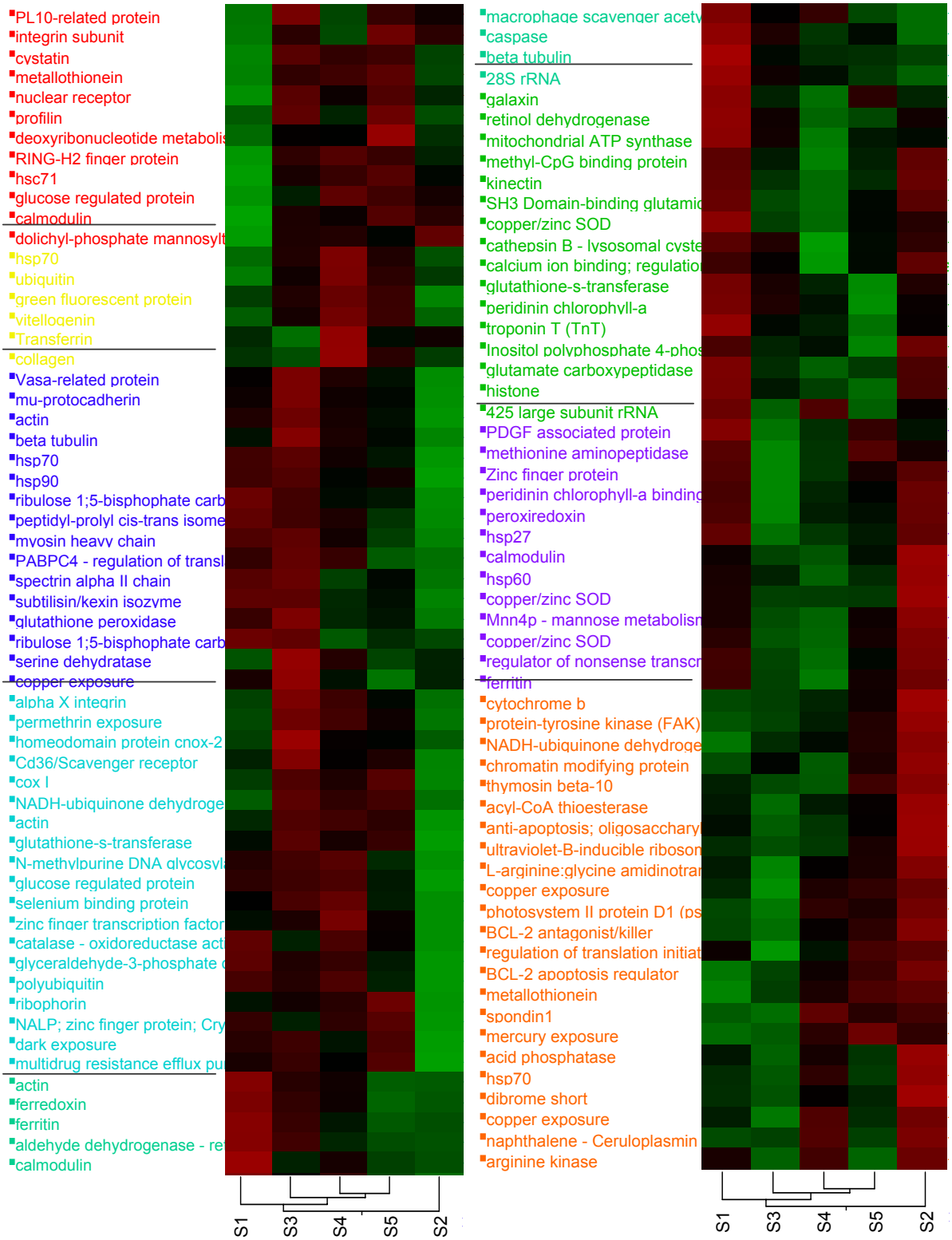


Figure A.5. Heat map of Ward's hierarchical clustering analysis of the deviations from the StdLsmean produced by the ANOVA between sites. Eight clusters of genes and three clusters of dates with similar profiles were generated. Red indicates a value > StdLSmean, green indicates a value < StdLSmean, and black is not different from the mean.

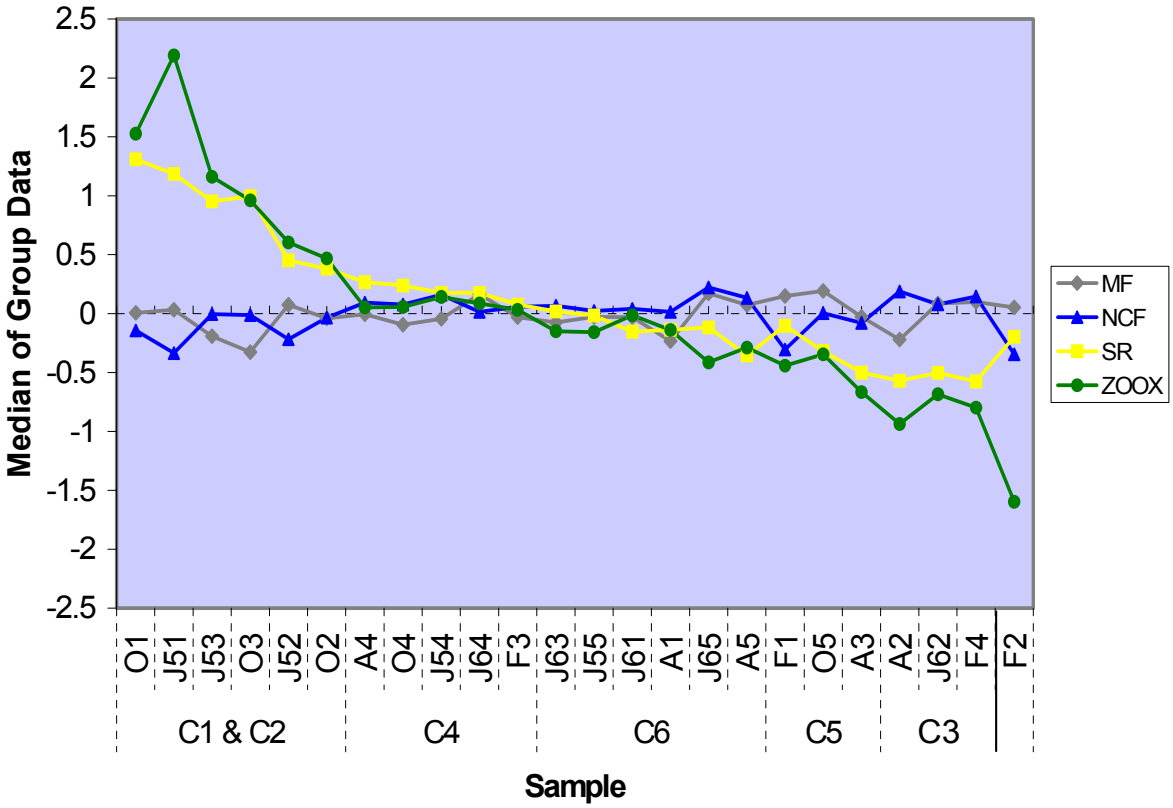


Figure A.6. Overview of response by group. Genes within functional categories were further grouped based on their overall role in the cell. These groups include normal cellular functioning genes (NCF), multifunctional genes (MF), genes involved in the stress response (SR), and symbiont specific genes (ZOOX). The median values of deviation from the StdLSmeans for genes within a group were graphed by sample.

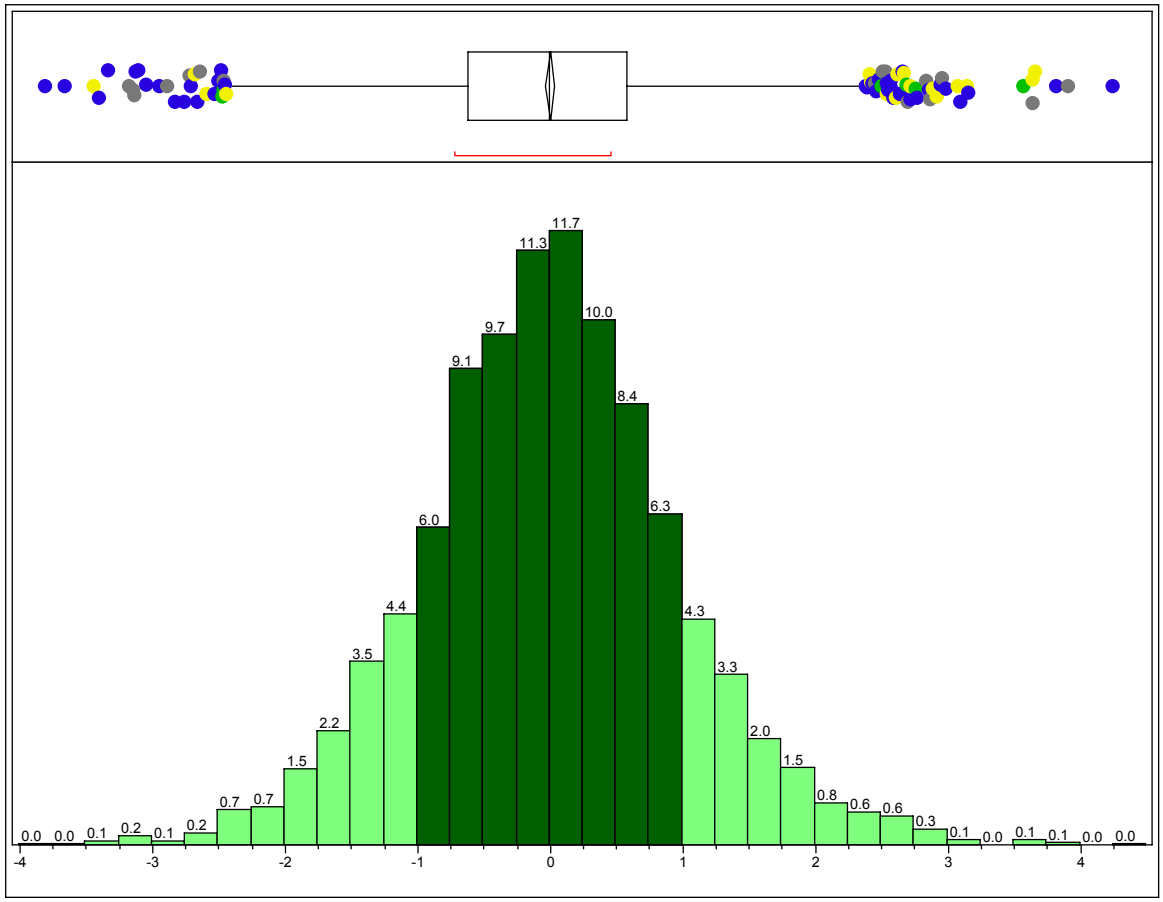


Figure A.7. Histogram distribution and outlier box plot of the deviations from the StdLsmean for each significant gene produced by the multivariate ANOVA. Most genes (72.5%) fall within +/- 1.0 of the StdLsmean and are indicated by the dark green bars. Outlier genes are indicated by colored dots; blue = normal cellular functioning genes (NCF), grey = multifunctional genes (MF); yellow = stress response genes (SR) and green = symbiont specific genes (ZOOX).

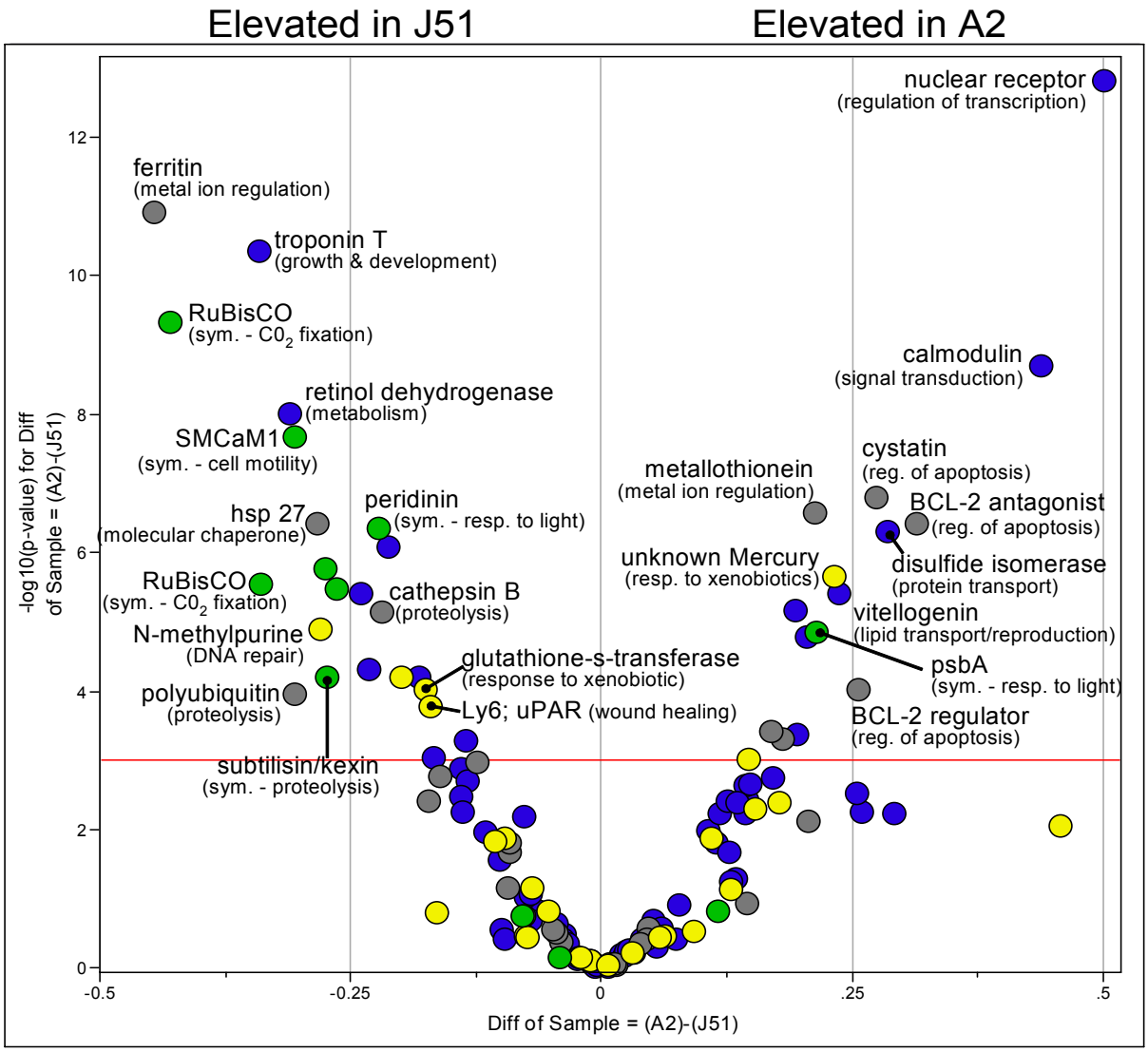


Figure A.8. Volcano Plot. Graph of genes that are significantly different between coral collected from site 2 in August (A2) and coral from site 1, June 2005 (J51). The X-axis is the difference in level of expression of a gene between the samples. The Y-axis is the -log10(p-value) that indicates the degree of significance (higher numbers = greater significance). The color of the circle indicates the functional group: blue = NCF (normal cellular function), grey = MF (multifunctional), yellow = SR (stress response) and green = ZOOX (symbiont specific). The dashed, red horizontal line is the cut-off for the false discovery rate (Q = 10). *Due to the large number of comparisons produced by this analysis (276) this is the only representative graph included in this report.*

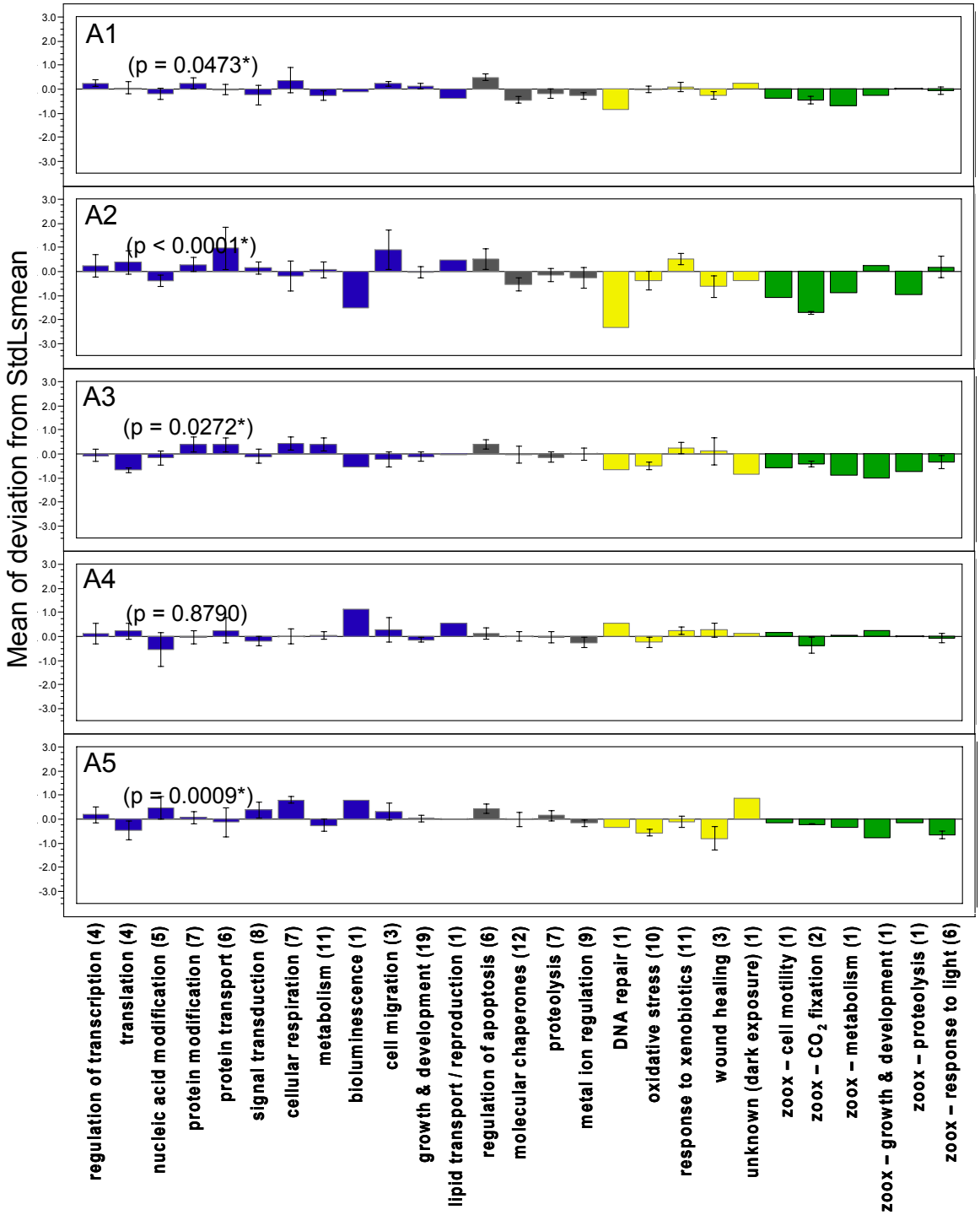


Figure A.9. Functional Response. Deviations from the standardized least squares mean (StdLsmean = 0) averaged between significant genes by functional category. Bars indicate standard error. Significant differences between categories are indicated by p-value (Welch ANOVA). The number of genes represented in each category is listed in parentheses after the category heading. Blue bars are categories in the normal cellular functioning group (NCF), grey bars represent multifunctional genes (MF), yellow bars indicate the stress response group (SR), green bars indicate symbiont-specific group (ZOOX).

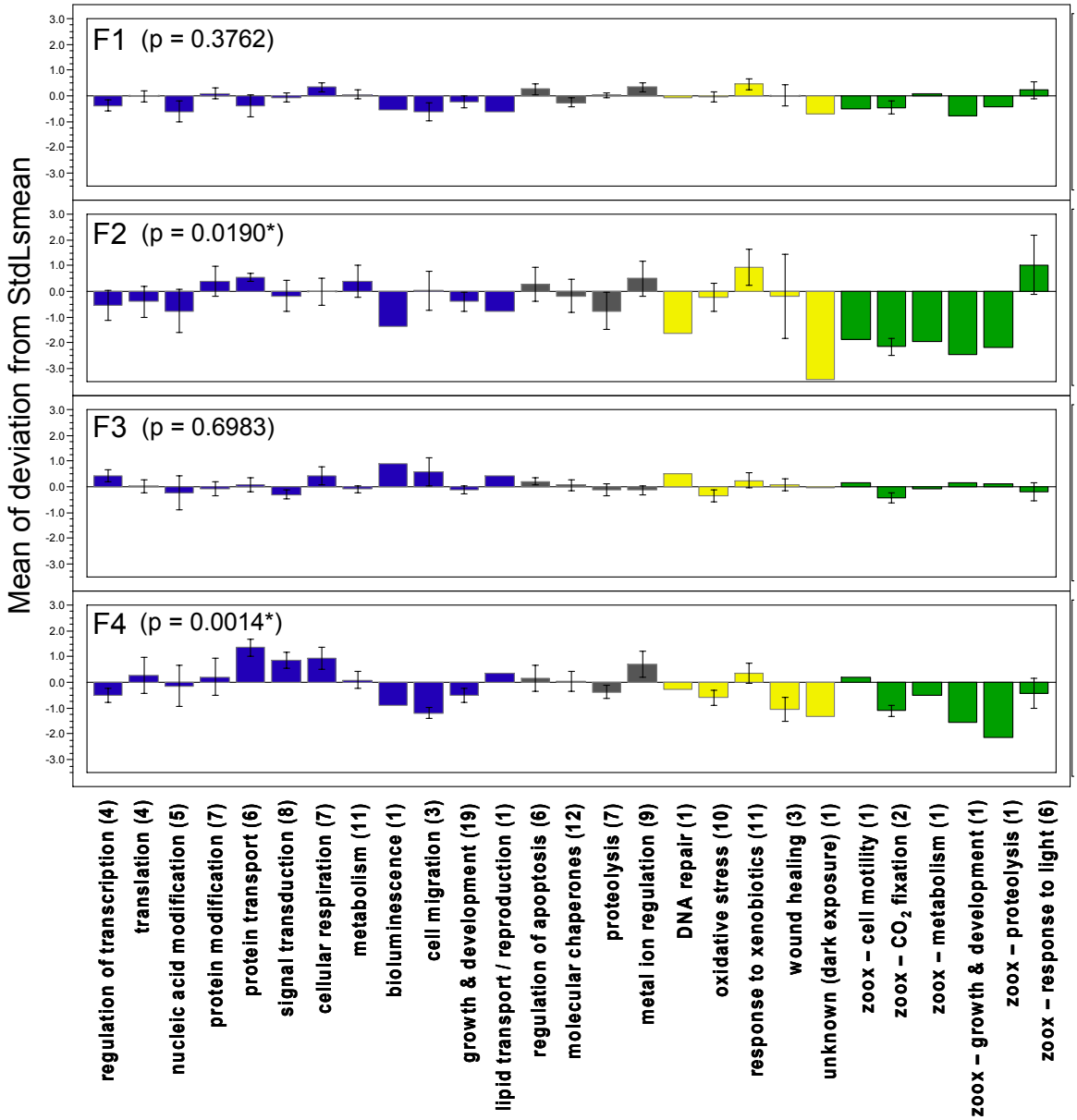
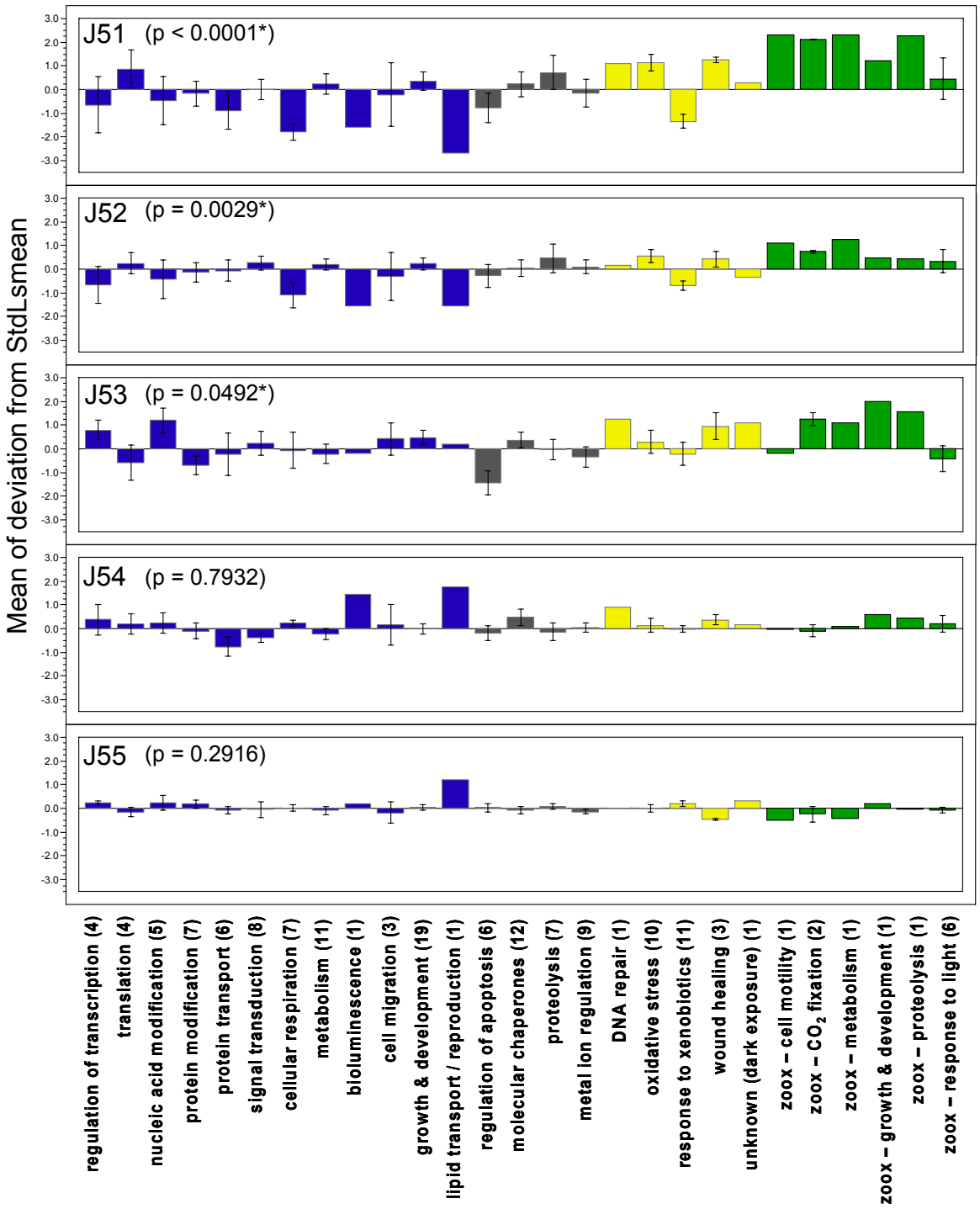


Figure A.9 (continued).



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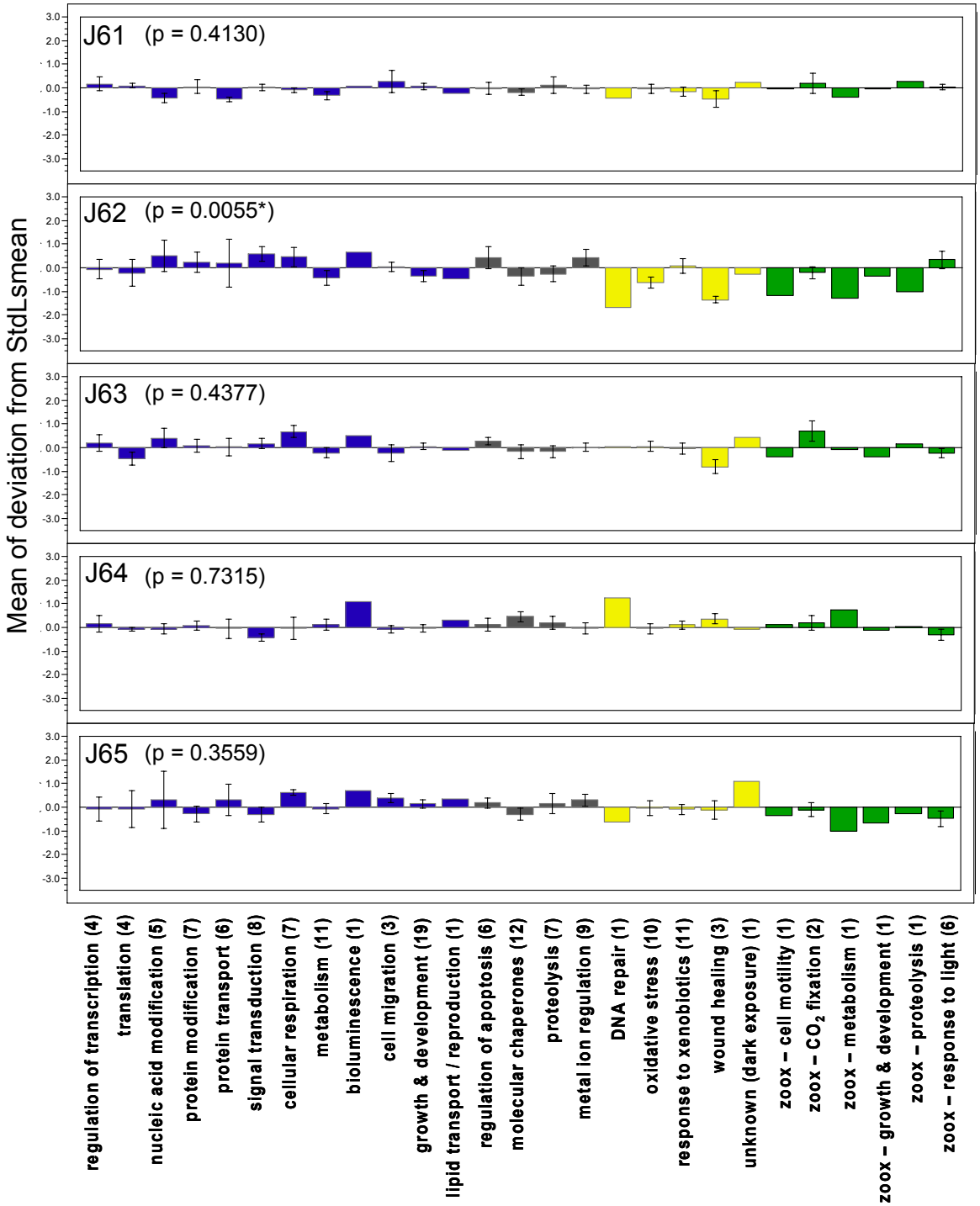

Figure A.9 (continued).

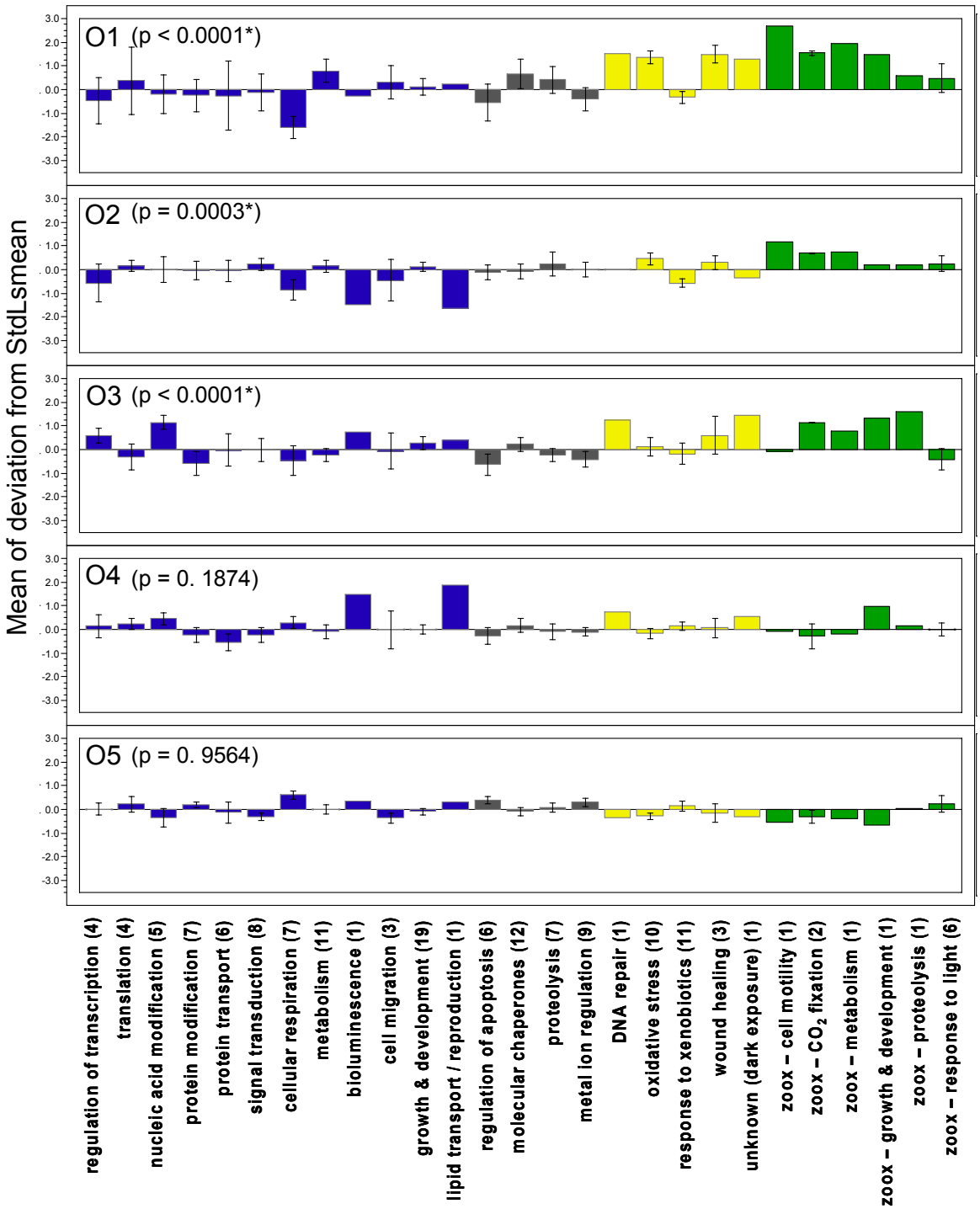

Figure A.9 (continued).

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